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THE APPLICATION OF SEROLOGICAL METHODS TO THE DIFFERENTIATION OF CLOSELY RELATED SMUT FUNGI[1]

BY E. C. BECK[2]

Abstract

Serological methods were applied in an attempt to differentiate a number of closely related members of the family Ustilaginaceae. The results of two series of reciprocal precipitin-ring tests showed that different genera and species of the same family were satisfactorily differentiated by this technique; so also were compatible cultures of the same species, where no detectable differences existed, other than the necessity of the haploid counterparts being brought together on the appropriate host plant to induce the diploid phase, and subsequent infection of the host. A parent culture and its mutant that were different morphologically but alike in their pathogenicity, were the only ones that could not be differentiated by this technique. Reciprocal absorption tests were applied to these two fungi, but the powder of either culture absorbed the antibodies of both from the immune sera. Optimal proportions of antigen and antibody were determined, but could not be applied in absorption tests because of the dilution of antisera. Agglutination tests were attempted but were unfruitful.

Introduction

Immunological studies have contributed a fund of information to perplexing questions of taxonomic relationships in groups of fungi and higher plants, where forms so closely resemble one another as to cause doubt regarding their relation. In view of the fact that some of the more recent serological investigations have thrown new light on the difficulties encountered in former studies, it was decided to reinvestigate the usefulness of the precipitin-ring test as an additional taxonomic criterion in differentiating a group of closely related smut fungi, employing, wherever possible, improvements in technique as reported in the literature. The smuts were chosen as test organisms because while they are highly obligate in their parasitic relations, many of them can be cultured on artificial media in their haploid stage. Within the group, therefore, it is possible to choose as experimental material members related in widely different degree, including different genera, species, physiological forms and even sexually compatible haplonts of heterothallic species. The precipitin-ring test was undertaken because of the encouraging results secured in earlier work (5), and because of the possibility that it might furnish some information with regard to the standardization of components to be used in other serological tests, applied subsequently.

[1] *Manuscript received July 13, 1938.*
Co-operative contribution from the Department of Botany and the School of Hygiene, University of Toronto, Ontario. Part of a thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in the University of Toronto.

[2] *Professor of Bacteriology, Ontario Agricultural College, Guelph, Ont. Formerly lecturer, Department of Botany, University of Toronto.*

The Precipitin-ring Test

Materials and Methods

The cultures employed were all members of the family Ustilaginaceae, representing different genera, species, compatible cultures* from the same chlamydospore, and one mutant and its parent culture. *Ustilago avenae* (Pers.) Jensen, *Sorosporium reilianum* (Kuhn) McAlpine, and *Sphacelotheca sorghi* (Lk.) Clinton, were procured from the Baarn Type Culture Collection, Baarn, Holland. *Ustilago zeae* (Beckm.) Ung. and *U. hordei* (Pers.) K. & S. were supplied through the courtesy of Dr. J. J. Christensen, Minnesota University. The three cultures of *U. zeae* were isolated from a single chlamydospore, as were the two cultures of *U. hordei*. *U. hordei* C. was a mutant from *U. hordei* B.

The medium recommended by Stakmann *et al.* (35)† as being especially suitable for the growth of this group of fungi was used throughout the experiments. Tests conducted to determine the most suitable pH of the medium indicated that the cultures were extremely tolerant with respect to pH and gave abundant growth on media adjusted to a range of pH 4.2 to 10. The optimum was approximately pH 6.8, and the medium did not need readjustment. All cultures were grown in eight-ounce medicine bottles containing about 30 cc. of the medium, which had been allowed to solidify over the maximum surface, and were incubated at room temperature for three to four weeks. Petri-dish cultures were found to be unsatisfactory because of the rapid drying of the medium, the frequency of contamination during such a long incubation period, and the relatively small yield of material. Fungal mats were peeled from the surface of the agar by means of a stout hooked needle, in such a way as to avoid bringing with them particles of the medium. Where this was unavoidable, the mats were scraped with a clean scalpel. The fungal mats were piled loosely in open Petri dishes and were dried in desiccators over sulphuric acid. Later, it was found more satisfactory to dry them at 45° C. in an incubator containing a large dish of anhydrous calcium chloride. The dry, crisp material was then ground in chemically clean mortars by means of a hand pestle, until fine enough to pass through a sieve of 100 mm. mesh. The powders were stored in sealed containers in the refrigerator. Powders prepared in this way have been kept for years without deterioration. The average net yield of dried material from a culture varied from 0.069 gm. to 0.935 gm.

Preparation of the Extracts

Injection fluids used for immunization were prepared by extracting 0.3 gm. of the dry powder in 10 cc. of 0.85% sodium chloride solution for 18 to 24 hr. in the refrigerator, after which the whole was centrifuged; the clear supernatant

** Cultures which when brought together on a suitable host will give rise to the diploid phase and produce infection.*

† Potato extract from 400 gm. of potatoes in 1 litre of water; 1.8% agar; 1% dextrose; 1% sucrose; and 1% malt extract.

fluid was used for intravenous injection, the sediment was resuspended in 10 cc. of saline and used concomitantly for intraperitoneal injections.

Antigens were prepared by extracting 0.3 gm. of the dry powder in 15 cc. of saline for 18 to 24 hr. in the refrigerator, followed by centrifuging until the supernatant fluid was crystal clear. Such fluids, which constituted the stock antigens, had a dilution of 1 : 50, and from them were prepared serial dilutions.

Injection fluids and antigens prepared in this manner were tested for the presence of proteins by the biuret test, and all gave a positive reaction.

Immunization of Rabbits

A small amount of blood was drawn from a marginal ear vein of each rabbit prior to immunization, and tested against the extract that was to be used later for injection. Since these normal sera were negative, the protocols of the tests have been omitted.

Immunization was accomplished by administering 2 cc. of the supernatant extract intravenously plus 3 cc. of the resuspended powder intraperitoneally, daily for three days. This was followed by a rest period of four days, after which the same treatment was repeated for three days. The animals were then rested for nine days, starved, and bled from the ear vein on the tenth day. From 30 to 50 cc. of blood was drawn, and the samples were stored in the refrigerator overnight. The next morning they were centrifuged, and the serum was removed to sterile vaccine bottles.

Setting up and Reading the Tests

Small precipitin tubes 10 mm. by 750 mm. were used, in order to economize with the antiserum. All glassware was cleaned in strong cleaning solution* before use. Saline used for dilution purposes and for controls was freshly prepared, as were the antigens. Undiluted antiserum (0.1 cc.) was used throughout the tests. Antigen dilutions were prepared in separate tubes, and by means of separate capillary pipettes, 0.1 cc. of the appropriate antigen dilution was carefully run over the same amount of serum.

Saline plus antigen and serum plus saline controls were included in all tests, but since they were negative throughout, they have been omitted from the tables.

All racks of tubes were incubated for one hour in a constant temperature water-bath at 45° C., after which they were read in a specially lighted chamber. Shorter periods of incubation were found to be unsatisfactory, since some of the reactions appeared more slowly than others. Reading the tests a second time after storing them in the refrigerator overnight was also unsatisfactory, because the diffusion of the components interfered with the reading.

In recording the results, "4" was used to indicate the maximum reaction, and in such instances there was not a clearly defined precipitin "ring" at the junction of the serum and antigen, but an opaque, fine and uniform preci-

* *Potassium dichromate saturated solution, 500 cc.; crude concentrated sulphuric acid, 800 cc.*

pitate throughout the mass; "3" indicates the presence of a continuous ring at the interphase, while "2" and "1" represent a definite ring less perfectly expressed. The sign ± is employed to indicate a doubtful reaction which could not be considered as a typical positive, although in a few instances such might have become positive with prolonged incubation.

The titre of an antiserum is taken as the highest dilution in which a continuous precipitin ring occurs between undiluted antiserum and its homologous antigen.

Results of the Precipitin Tests

The results of these tests are presented in Table I, from which it is seen that potent antisera were produced for all extracts, the lowest titre being 1 : 3200, while six antisera possessed a titre of 1 : 12,800. Not only were the antisera high in titre, but they showed a high degree of specificity. The three extracts of monosporidial cultures of *Ustilago zeae* were distinguished as separate serological entities; so also were *U. hordei* A and B, which were also monosporidial cultures from a single chlamydospore. Extracts of the mutant culture *U. hordei* C were indistinguishable from the parent culture *U. hordei* B, when tested reciprocally by this technique. The three antisera of *U. zeae* gave strong group reactions with *U. avenae* antigen, but the antiserum of *U. avenae* was not reciprocally reactive with the antigens of *U. zeae*. The antiserum of *Sorosporium reilianum* gave marked group reactions with most of the antigens of other cultures, but the antisera of these cultures were not proportionately reactive with the antigen of *Sorosporium*.

Interpreting these results on the basis of the conventional reading of the precipitin and agglutination reactions in general practice, it is concluded that the differences between titres and non-specific reactions are great enough to serve as a basis for identification of the members of this group of fungi. *Ustilago hordei* B and its mutant were the only exceptions.

Supplementary Tests

Second Series of Precipitin Tests

In this series of tests, modifications were made in the preparation of the injection fluids and antigens, and in the immunization of the rabbits. In all other respects the procedure was similar to that already described.

The injection fluids were prepared by regrinding 0.3 gm. of the powder for about 10 min. with small amounts of saline, until 6 cc. had been incorporated. The mortar was rinsed with 4 cc. of saline. The mixture was allowed to extract in the refrigerator for 18 to 24 hr., then centrifuged. The supernatant fluids remained turbid.

Antigens used in this series were prepared by regrinding 0.3 gm. of powder in 15 cc. of saline, followed by extraction and centrifuging as above. These extracts could not be used as antigens until they had been filtered through Seitz bacteria-proof filters to render them crystal clear.

The turbid fluids gave much more marked biuret reactions than the clear extracts. Extraction of the powders was also attempted with glycerine,

TABLE I

RESULTS OF FIRST SERIES OF PRECIPITIN TESTS

Antigens	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12,800	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12,800	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12,800
	U. zeae A antiserum									*U. zeae* B antiserum									*U. zeae* C antiserum								
U. zeae A	2	1	1	1	1	1	1	—	—	3	2	1	1	1	—	—	—	—	4	3	2	1	2	1	—	—	—
U. zeae B	1	1	—	—	—	—	—	—	—	4	1	1	1	1	3	3	3	1	4	3	2	2	1	±	—	—	—
U. zeae C	1	1	—	—	—	—	—	—	—	4	3	3	1	—	—	—	—	—	4	3	3	3	1	1	1	2	1
U. hordei A	1	1	—	—	—	—	—	—	—	2	1	±	—	—	—	—	—	—	4	3	3	1	±	—	—	—	—
U. hordei B	2	1	±	—	—	—	—	—	—	3	1	±	—	—	—	—	—	—	4	1	1	1	—	—	—	—	—
U. hordei C	3	1	1	—	—	—	—	—	—	4	1	±	1	—	—	—	—	—	4	1	1	±	—	—	—	—	—
U. avenae	4	2	1	1	—	—	—	—	—	4	3	2	1	1	—	—	—	—	4	3	3	2	1	1	—	—	—
S. reilianum	2	1	1	—	—	—	—	—	—	4	1	1	—	—	—	—	—	—	3	±	±	1	1	—	—	—	—
S. sorghi	1	1	1	±	±	—	—	—	—	1	—	—	—	—	—	—	—	—	4	1	1	1	±	—	—	—	—
	U. hordei A antiserum									*U. hordei* B antiserum									*U. hordei* C antiserum								
U. zeae A	4	1	1	1	—	—	—	—	—	4	1	—	—	—	—	—	—	—	2	1	±	±	±	±	±	—	—
U. zeae B	4	2	1	—	—	—	—	—	—	4	1	—	—	—	—	—	—	—	3	2	1	—	±	±	—	—	—
U. zeae C	4	2	1	—	—	—	—	—	—	4	±	±	—	—	—	—	—	—	3	2	1	±	±	±	±	—	—
U. hordei A	4	3	3	1	1	1	1	±	—	4	3	2	2	1	—	—	—	—	2	1	1	1	1	1	—	—	—

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TABLE I—*Concluded*

RESULTS OF FIRST SERIES OF PRECIPITIN TESTS—*Concluded*

Antigens	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12,800	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12,800	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	1 : 3200	1 : 6400	1 : 12,800
	U. hordei A antiserum—*Con.*									*U. hordei* B antiserum—*Con.*									*U. hordei* C antiserum—*Con.*								
U. hordei B	4	2	2	1	1	—	—	—	—	4	2	2	2	2	1	1	1	1	4	2	2	1	1	1	1	±	—
U. hordei C	4	3	3	1	1	±	—	—	—	4	2	2	1	±	1	1	1	±	4	2	2	2	1	1	1	1	1
U. avenae	4	3	1	1	—	—	—	—	—	4	2	1	1	±	—	—	—	—	4	3	1	—	—	—	—	—	—
S. reilianum	4	1	1	—	—	—	—	—	—	4	1	—	—	—	—	—	—	—	2	1	1	—	—	—	—	—	—
S. sorghi	4	2	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2	1	±	±	—	—	—	—	—
	U. avenae antiserum									*Sorosporium reilianum* antiserum									*Sphacelotheca sorghi* antiserum								
U. zeae A	3	2	1	—	—	—	—	—	—	4	2	1	1	—	—	—	—	—	4	1	1	—	—	—	—	—	—
U. zeae B	2	1	1	—	—	—	—	—	—	4	3	2	1	—	—	—	—	—	3	—	—	—	—	—	—	—	—
U. zeae C	2	1	1	—	—	—	—	—	—	3	2	2	1	1	—	—	—	—	3	—	1	—	—	—	—	—	—
U. hordei A	1	1	1	—	—	—	—	—	—	3	2	2	2	2	1	—	—	—	4	—	—	—	—	—	—	—	—
U. hordei B	3	2	1	—	—	—	—	—	—	4	3	2	2	2	1	—	—	—	4	—	1	±	—	—	—	—	—
U. hordei C	3	2	1	—	—	—	—	—	—	4	2	2	1	1	—	—	—	—	4	1	1	±	—	—	—	—	—
U. avenae	4	4	3	2	2	2	1	1	1	4	3	3	2	1	1	—	—	—	4	3	1	±	—	—	—	—	—
S. reilianum	3	1	1	—	—	—	—	—	—	4	3	2	2	1	1	1	1	±	4	1	—	—	—	—	—	—	—
S. sorghi	2	1	1	—	—	—	—	—	—	4	1	1	1	1	—	—	—	—	4	2	2	2	2	2	1	1	1

All antisera were used undiluted.

4 = very heavy precipitate throughout serum and antigen; 3 = a continuous ring at the interphase; 2 and 1 = a definite ring less perfectly expressed; ± = doubtful reaction.

hypertonic saline (2.55% sodium chloride), and 1% sodium carbonate, but these methods were abandoned since they offered some disadvantages without compensating advantages.

Immunization

The turbid extract (1 cc.) was used as the initial intravenous injection and no intraperitoneal injection was made. Of seven rabbits treated in this way, five succumbed within 48 hr. Another group of animals was started with an initial intravenous dose of 0.25 cc. of the turbid fluid, followed by five subsequent doses of 0.5 and 1.0 cc., and three injections of 1.5 cc. each, without intraperitoneal injections. After resting the animals for four days, four intraperitoneal injections of the resuspended sediment were given in 2-cc. amounts. This was followed by the procedure already described.

Results

The results of this series of precipitin tests are given in Table II, from which it will be seen that, while the results are parallel to and in general agreement with the first series, the titres throughout are much lower, as are the non-specific or group reactions. Eight of the nine antisera possessed a titre of 1 : 1600 and one titre of 1 : 3200, while the highest group reaction occurred in an antigen dilution of 1 : 400, and the degree of specificity is approximately the same in the two series. It would seem that the difference in the immunizing procedure might account, in part at least, for the fall in titre in the second series of tests, since the animals lost considerable weight during immunization.

Additional Refinements in Technique

In order to differentiate still more sharply the components of the series studied, the following three refinements in technique were attempted; first, to increase the antibody content of immune sera; second, to determine the optimal proportions of antigen and antibody; and third, reciprocal absorption tests.

Attempts to Increase the Antibody Content of Immune Sera

Various workers have noted a marked rise in the titre of an antiserum resulting from a single injection of the immunizing fluid administered several days after the last injection of the immunizing series. It was felt that if this condition could be achieved, sharper differentiation of the various fungi might result. In order to test the possibility, seven of the rabbits used in the second series of tests were each given one intraperitoneal injection, consisting of 2.5 cc. of the resuspended appropriate fungus powder. The injection was given 12 days after the last injection of the regular series, or two days after bleeding for the regular tests. After a rest period of 12 days, sufficient blood was drawn from the ear vein to test against the homologous antigen. The results given in Table III indicate that no secondary stimulation resulted. They do, however, confirm repeated observations with regard to the rapid fall in titre of the antisera, both *in vivo* and *in vitro*. An examination of the data contained in Table III reveals a reduction in titre from 1 : 1600 to 1 : 200 after 24 days "storage *in vivo*".

TABLE II

RESULTS OF SECOND SERIES OF PRECIPITIN TESTS

Antigens	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800
	U. zeae A antiserum									*U. zeae* B antiserum									*U. zeae* C antiserum								
U. zeae A	4	4	3	2	1	1	±	±	±	3	1	—	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—
U. zeae B	2	1	—	—	—	—	—	—	—	4	3	2	1	1	1	±	±	—	4	3	2	1	±	—	—	—	—
U. zeae C	4	3	2	1	—	—	—	—	—	2	2	1	—	—	—	—	—	—	4	4	3	2	1	1	—	—	—
U. hordei A	4	3	2	1	—	—	—	—	—	3	1	—	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—
U. hordei B	4	3	—	—	—	—	—	—	—	3	2	—	—	—	—	—	—	—	3	2	—	—	—	—	—	—	—
U. hordei C	3	1	—	—	—	—	—	—	—	3	1	—	—	—	—	—	—	—	3	2	—	—	—	—	—	—	—
U. avenae	3	2	1	—	—	—	—	—	—	2	1	—	—	—	—	—	—	—	4	3	—	—	—	—	—	—	—
S. reilianum	2	3	1	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	4	3	—	—	—	—	—	—	—
S. sorghi	4	3	2	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	4	3	1	—	—	—	—	—	—
	U. hordei A antiserum									*U. hordei* B antiserum									*U. hordei* C antiserum								
U. zeae A	3	2	1	—	—	—	—	—	—	3	2	1	±	—	—	—	—	—	2	1	—	—	—	—	—	—	—
U. zeae B	4	3	2	1	—	—	—	—	—	4	3	2	1	—	—	—	—	—	3	1	—	—	—	—	—	—	—
U. zeae C	3	2	1	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—	1	1	±	—	—	—	—	—	—
U. hordei A	4	3	2	1	1	1	±	—	—	2	1	±	—	±	—	—	—	—	3	2	1	1	1	—	—	—	—

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TABLE II—*Concluded*

RESULTS OF SECOND SERIES OF PRECIPITIN TESTS—*Concluded*

Antigens	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800
	U. hordei A antiserum—*Con.*									*U. hordei* B antiserum—*Con.*									*U. hordei* C antiserum—*Con.*								
U. hordei B	3	2	—	±	±	—	—	—	—	3	2	1	1	1	1	±	—	—	4	3	2	1	1	±	±	—	—
U. hordei C	3	2	—	—	—	—	—	—	—	4	3	1	1	1	1	±	±	—	4	3	2	1	1	1	1	—	—
U. avenae	3	2	1	1	—	—	—	—	—	4	3	2	1	—	—	—	—	—	4	3	2	—	—	—	—	—	—
S. reilianum	2	1	—	—	—	—	—	—	—	3	2	1	±	—	—	—	—	—	3	2	1	±	—	—	—	—	—
S. sorghi	3	—	—	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—	3	2	—	—	—	—	—	—	—
	U. avenae antiserum									*Sorosporium reilianum* antiserum									*Sphacelotheca sorghi* antiserum								
U. zeae A	2	1	±	—	—	—	—	—	—	3	1	—	—	—	—	—	—	—	2	1	—	—	—	—	—	—	—
U. zeae B	2	1	±	—	—	—	—	—	—	3	1	—	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—
U. zeae C	3	2	1	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—
U. hordei A	3	2	1	—	—	—	—	—	—	2	1	—	—	—	—	—	—	—	2	1	—	—	—	—	—	—	—
U. hordei B	3	2	1	±	—	—	—	—	—	3	2	1	—	—	—	—	—	—	2	1	—	—	—	—	—	—	—
U. hordei C	3	2	1	±	—	—	—	—	—	2	±	±	—	—	—	—	—	—	3	2	1	—	—	—	—	—	—
U. avenae	4	3	2	1	1	1	±	—	—	4	3	2	1	±	—	—	—	—	2	1	±	—	—	—	—	—	—
S. reilianum	3	2	1	—	—	—	—	—	—	3	2	1	1	1	1	—	—	—	2	1	—	—	—	—	—	—	—
S. sorghi	2	1	—	—	—	—	—	—	—	3	—	—	—	—	—	—	—	—	4	3	2	1	1	1	±	±	—

All antisera were used undiluted.

4 = very heavy precipitate throughout serum and antigen; 3 = a continuous ring at the interphase; 2 and 1 = a definite ring less perfectly expressed; ± = doubtful reaction.

TABLE III

ATTEMPT TO INCREASE SERUM TITRE BY SECONDARY STIMULATION

Antisera + homologous antigens

Antisera	Antigen dilutions						Original titre
	1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600	
U. zeae A	3	2	1	—	—	—	1 : 1600
U. zeae B	3	2	±	—	—	—	1 : 1600
U. zeae C	3	2	1	—	—	—	1 : 1600
U. hordei A	3	2	2	±	—	—	1 : 1600
U. hordei B	3	2	1	—	—	—	1 : 1600
U. hordei C	2	1	1	—	—	—	1 : 3200
S. reilianum	3	2	1	—	—	—	1 : 1600

Optimal Proportions of Antigen and Antibody

Before conducting reciprocal absorption tests to determine the serological relation of *U. hordei* B and its mutant *U. hordei* C, it was deemed advisable to determine the optimal proportion of antigen to undiluted antiserum, in the hope that such tests would furnish some guide towards the technique of effective absorption. Dean and Webb (11) pointed out the usefulness of the method for the quantitative estimation of either antigen or antibody, and Ramon (33) demonstrated the value of the test in the titration of diphtheria antitoxin. Smith (34), employing this technique, concluded that *in vitro* tests for determining the potency of antipneumococcus serum were more accurate and more rapid than the laborious and expensive animal tests. In conducting absorption tests it is important to know the optimal proportions of the reagents, since most immunological reactions with a relative excess of one or other of the test fluids may show "specific inhibition" preventing the visible expression of the reaction. This phenomenon has been attributed by Opie (30, 31) to an excess of antigen, which dissolves the precipitate formed by the union of precipitin and its antigen.

The antisera were used undiluted against graded dilutions of antigen made up to the same volume of serum with 0.85% saline, as indicated in Table IV. Saline and antigen were added to the tubes first and mixed, followed by 0.5 cc. of the antiserum, which was carefully run down the side of the tube. The tubes were left undisturbed at room temperature until the "critical tube" or "indicator tube" was noted. The tests were then incubated at 45° C. for one hour before making the final readings. On addition of *U. hordei* B serum to graded dilutions of the homologous antigen, precipitation occurred in one and one-half minutes in the tube containing 0.05 cc. of antigen, or in an antigen dilution of 1 : 10, while precipitates formed more slowly on either side of this dilution. When *U. hordei* C antiserum was added to 0.4 cc. of its antigen (1 : 1.25 dilution), precipitation occurred at once. The optimal proportions of antigen and antibody (as indicated by the "critical tube", using dilutions of 1:50 stock antigen) were 1 : 10 and 1 : 1.25 respectively,

and when these dilutions were departed from, in the direction of either antigen or antibody excess, precipitation took place more slowly. Dean and Webb pointed out that most rapid precipitation occurred in the mixture in which antigen and antibody were present in such amounts that none of either reagent remained uncombined, or that only a trace remained detectable in the supernatant fluid.

TABLE IV

OPTIMAL PROPORTIONS OF ANTIGEN AND ANTIBODY

	Schedule									
									Controls	
Tubes	1	2	3	4	5	6	7	8	9	10
Saline	0	.1	.2	.3	.4	.45	.47	.49	.5	.5
Antigen	.5	.4	.3	.2	.1	.05	.03	.01	.5	.0
Serum	.5	.5	.5	.5	.5	.5	.5	.5	0	.5
	Reactions									
U. hordei B antigen, 1 : 50 *U. hordei* B serum, undiluted	–	–	–	2	3	3*	3	2	–	–
U. hordei C antigen, 1 : 50 *U. hordei* C serum, undiluted	3	3†	3	2	2	2	–	–	–	–

* *"Indicator tube" appeared in 1½ min.*

† *"Indicator tube" immediate reaction.*

Reciprocal Absorption

This test was employed as a final means of determining whether *U. hordei* B and its mutant *U. hordei* C were serologically identical, since they have proved to be more or less reciprocally reactive to the same degree, as exemplified by the precipitin-ring test.

The optimal proportions of antigen and antibody, as previously determined, were found to be unsatisfactory for application in the absorption tests, since the dilution of the serum by the antigen rendered it unsuitable for the ring test. It was also found necessary to employ the powder instead of the powder extract, in order to effect satisfactory absorption of the antibodies. To determine the actual relation of the mutant culture and its parent, absorption was conducted in the following manner: to 0.4 gm. of the fungus powder, 1.6 cc. of saline was added. These were ground together in a mortar, removed to the refrigerator and allowed to extract for 18 to 24 hr., after which the suspension was added to 2 cc. of the serum to be absorbed. These serum-extract mixtures were incubated in a water-bath at 56° C. for 2 hr. with frequent shaking. They were then stored in the refrigerator for 18 hr. before being centrifuged, in order to secure clear serum. Such treatment did not, however, produce the crystal-clear serum needed for the tests, and it was found necessary to filter the supernatant fluid through Seitz

bacteria-proof filters. During absorption, a fine permanent turbidity appeared which was identical with a "4" precipitin reaction.

TABLE V

RECIPROCAL ABSORPTION OF PRECIPITINS

From *U. hordei* B antiserum and *U. hordei* C antiserum

Antiserum	Suspension	Antigen	Antigen dilutions					
			1 : 50	1 : 100	1 : 200	1 : 400	1 : 800	1 : 1600
U. hordei B absorbed by *U. hordei* B *vs. U. hordei* B			–	–	–	–	–	–
U. hordei B absorbed by *U. hordei* B *vs. U. hordei* C			–	–	–	–	–	–
U. hordei B absorbed by *U. hordei* C *vs. U. hordei* B			–	–	–	–	–	–
U. hordei B absorbed by *U. hordei* C *vs. U. hordei* C			–	–	–	–	–	–
U. hordei C absorbed by *U. hordei* B *vs. U. hordei* B			–	–	–	–	–	–
U. hordei C absorbed by *U. hordei* B *vs. U. hordei* C			–	–	–	–	–	–
U. hordei C absorbed by *U. hordei* C *vs. U. hordei* B			–	–	–	–	–	–
U. hordei C absorbed by *U. hordei* C *vs. U. hordei* C			–	–	–	–	–	–
Controls								
U. hordei B *vs. U. hordes* B		Antigens	4	3	3	2	1	±
U. hordei B *vs. U. hordei* C			4	3	3	2	1	1
U. hordei C *vs. U. hordei* B			4	3	2	1	±	–
U. hordei C *vs. U. hordei* C			4	3	2	2	1	1
Diluted antiserum—1 : 1								
U. hordei C *vs. U. hordei* C		Antigens	2	1	1	±	–	–
U. hordei B *vs. U. hordei* B			2	1	1	–	–	–

As a precaution against misinterpretation resulting from dilution of the antiserum by the absorbing material, serum diluted with an equal volume of saline was tested with standard serially diluted antigen. The reduction in reaction of serum by dilution is shown in Table V.* The schedule of reciprocal absorption tests is also given in Table V.

It was concluded from the results of these tests that these antisera were totally non-specific, being completely absorbed reciprocally by the antigens, while the controls remained positive. Or, in other words, these two sera contained major antibodies indicating that the two cultures were serologically identical. If, then, these two cultures are to be considered as separate entities, some criterion other than the precipitin-ring and absorption tests must be employed.

The Agglutination Test

In taxonomic studies dealing with phytopathogenic bacteria, the agglutination technique has been applied with results that would seem to justify its use as a "standard" criterion in identification and classification. However,

* *It should be remembered that absorption sometimes reduces the titre of a serum for its homologous antigen.*

a more careful perusal of the literature reveals many instances in which the test was not suitable.

The relative simplicity of the test is so advantageous that repeated attempts have been made by the author to apply the method in studies of fungus relations.

The preparation of a suitable antigen has been the chief obstacle in the application of this test, but the antigen as prepared in the second series of precipitin tests, before filtration to clear it, seemed to present a suspension analogous to a bacterial suspension, insofar at least as it remained uniformly turbid.

To serially diluted antiserum an equal volume of undiluted antigen (stock solution 1:50) was added. The tubes were shaken and incubated at 37° C. for one hour, after which they were read in a specially lighted rack as used for reading the precipitin tests. There was no indication of a typical agglutination reaction, all tubes showed varying degrees of uniform turbidity which remained in a state of permanent suspension. The Widal or microscopical agglutination test was equally unfruitful. Such results were not altogether surprising after considering the failure to obtain a satisfactory reading from precipitin-shake tests, in order to interpret the results as in the Kahn test.

The precipitate resulting from the precipitin reaction always remained in a more or less permanent state of suspension. In some instances, however, after standing for prolonged periods, precipitates disappeared altogether, as though they had been absorbed. It is possible that contamination and proteolytic enzymes might have played a part in such circumstances, or possibly the presence of an excess of antigen was responsible.

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VARIETAL DIFFERENCES IN BARLEYS AND MALTS

II. SACCHARIFYING ACTIVITIES OF BARLEYS AND MALTS AND THE CORRELATIONS BETWEEN THEM[1]

By Henry R. Sallans[2] and J. Ansel Anderson[2]

Abstract

Determinations of free and total saccharifying activity were made on 144 samples of barley, and free saccharifying activity (Lintner value) was also determined on kilned malts made from these barleys. The samples represent 12 varieties of barley grown at 12 widely separated experimental stations in Canada.

Varietal differences were demonstrated with respect to each determination. In total barley activity and malt activity, Olli was outstandingly high; the remaining six-rowed rough-awned varieties, Pontiac, Mensury Ott. 60, O.A.C. 21, and Peatland, and the smooth-awned variety Velvet, also yielded comparatively high values; the two-rowed variety Hannchen gave intermediate values; and the two-rowed varieties Victory and Charlottetown 80, and the remaining smooth-awned six-rowed varieties, Nobarb, Wisconsin 38, and particularly Regal, were low in activity. With respect to free barley activity the varieties fell in the same order with the exception of Olli, Peatland and Charlottetown 80 which gave very low values. These three varieties have only about 22% of total barley amylase in free form whereas figures for the other nine varieties range between 38 and 44%.

There is a close correlation ($r = 0.997$) between total barley activity as measured by the papain and hydrogen sulphide methods, the former giving rather higher values. Varieties that are high in total barley activity also tend to be high in malt activity (papain, $r = 0.904$; H_2S, $r = 0.868$). A similar relation exists between free barley activity and malt activity for nine of the varieties ($r = 0.971$), but if the three varieties having low percentages of free amylase are included the correlation is not significant ($r = 0.217$). Environment affects each property in essentially the same manner so that mean values for the different stations fall in much the same order for each determination and correlation coefficients for station means are all high.

The possible utility of determinations of total barley saccharifying activity for facilitating the selection of strains of good malting quality from collections of hybrid lines is discussed.

As a result of investigations made by Myrbäck (14, and earlier papers) it appears to be well established that the amylase activities, both free and total, of barley and malt are varietal characteristics. Data accumulated by others (2, 5, 6, 9, 12, 17, 18) who have investigated amylase activity in barley or malt, or both, provide considerable additional support for this hypothesis. On the other hand, Chrzaszcz and Sawicki (8), in a recent paper based on an extensive investigation of the subject, take issue with Myrbäck and summarize their findings by writing "Ein Einfluss der Gerstensorte auf die Amylasemenge konnte nicht festgestellt werden." Myrbäck and Örtenblad have since replied in convincing manner (16).

The investigation described in this paper yields additional evidence on the effect of variety on amylase activity. It was undertaken with the object of obtaining information on varietal differences in barley and malt saccharifying

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[2] *Biochemist, National Research Laboratories, Ottawa.*

activities amongst a representative set of the barley varieties grown in Canada. The work also forms part of a more comprehensive investigation designed for the collection of an adequate body of data for the statistical examination of the relations between various barley and malt properties, and the bearing of these on the problem of evaluating the malting qualities of new varieties of barley.

Materials

The barley samples used in the present investigation were described in detail in Part I of this series of papers (1). Briefly, they consist of 144 samples representing 12 varieties grown at each of 12 widely separated experimental stations in Canada. The varieties are listed in Table I and the stations in Table II.

Duplicate malts were made from each barley sample in the malting laboratory at the University of Manitoba. The equipment and methods used have already been described (3). In order to control systematic errors that might arise in malting a series of samples that required three months to complete, the malting was carried out in the following manner. Each batch of malt contained 12 samples representing the 12 varieties grown at one station. The samples were arranged in random order within batches and the batches were malted in random order. The first replicates were made in the first 12 batches and the second replicates in the succeeding 12 batches. It will be apparent that these methods provide for an unbiased comparison of the varieties.

Methods

The free saccharifying activity of malt, *i.e.*, the Lintner value, was determined by the ferricyanide modification of the official method of the American Society of Brewing Chemists (4), and the results are reported in the usual manner in degrees Lintner. The saccharifying activities of the barleys were determined by the same method, with certain necessary modifications that are described below, and the results are also reported in degrees Lintner.

Barley samples were ground, shortly before use, in a Wiley mill with a 1.0 mm. sieve, and were then carefully mixed. Extractions were made at 20° C. using a 2.5-gm. aliquot of barley meal and 50 ml. of water contained in a 200–220 ml., wide-neck, volumetric sugar flask. The ratio of meal to solution was thus identical with that used for determinations made on malt.

Extractions for the determination of free saccharifying activity were made with distilled water for 2.5 hr.

In determining total activity by the papain method a 21.5-hr. extraction was made with the addition of 0.5 gm. of Merck's papain (*cf.* 10). For the determination of total activity by the hydrogen sulphide method, the flask was fitted with a rubber stopper and a delivery tube with a short length of rubber tubing and a pinch-cock. After introducing and mixing the barley meal and water, the air above the solution was displaced with hydrogen sulphide and the flask was allowed to stand for 20 min., with occasional shaking,

under a pressure of gas equivalent to 16 in. of water. This process was repeated and the flask, still under pressure, was then closed by means of a pinch-cock and allowed to stand in a water bath at 20° C. for 20.5 hr. At the end of this time the extracts were filtered, the air above the filtrates was displaced with hydrogen sulphide, and the flasks were stoppered until just before the solution was pipetted for the diastasis (*cf.* 7).

As a check on the precision of the analyses, duplicate determinations were made on one-third of the barley samples. The standard deviations of the means of duplicate determinations proved to be:— 0.9° L. for free activity; 1.7° L. for total activity by papain; and 1.9° L. for total activity by hydrogen sulphide.

Duplicate malts were made from each barley and single determinations of saccharifying activity were made on each malt. An estimate of the combined sampling, malting and analytical errors is therefore available with respect to this determination. The standard deviation of the mean of determinations made on duplicate malts proved to be 3.7° L.

Significance of Properties Measured

Before discussing the results of the investigation it seems wise to consider the significance of the various measurements that were made. In this matter there is room for considerable difference of opinion and the authors feel it incumbent upon them to state theirs.

Barley contains considerable quantities of the saccharogenic β-amylase. Only part of this can be extracted with water. According to Myrbäck and his co-workers (15, 16) the remainder is bound in some manner to the protein, and can be liberated by the action of the proteolytic enzyme papain, or of compounds, such as hydrogen sulphide, that stimulate the action of proteolytic enzymes already present in the barley. On the other hand, Chrzaszcz and his co-workers (7, 8, and papers cited therein) consider this hypothesis inadequate to explain all the data obtained by extracting barley in the presence of various enzyme preparations and enzyme-stimulating substances. They believe that barley contains sisto- and eleuto-substances, which also play a part in the reactions. It is generally agreed, however, that during the germination process most of the latent or inactive β-amylase is liberated or activated so that a very large proportion of the β-amylase present in malt can be extracted with water.

According to current hypotheses (13, p. 98), barley contains no free dextrogenic α-amylase, or at most, only small quantities of it. On the other hand, malt contains considerable quantities of α-amylase together with all the β-amylase originally present in the barley. Whether the α-amylase, or some essential activator for it, is actually elaborated during the germination process, or whether it is present in barley in unextractable or inactive form, remains a moot question. In malt both enzymes are present largely in such form that they can be extracted with water, and since α-amylase also has some saccharifying activity (11), determinations of saccharifying activity

made with aqueous extracts of malt measure the combined activities of free α- and β-amylase.

With these hypotheses in mind we may now consider what the determinations used in the present investigation actually measure. It is obvious, without further argument, that the determination of free barley saccharifying activity provides a comparative measure of the free β-amylase present in the samples.

Some differences of opinion may well exist with respect to the interpretation of the results of determinations of total barley saccharifying activity by the papain and hydrogen sulphide methods used in the investigation. Chrzaszcz and his co-workers (8, and papers cited therein) obtained higher values by extracting with a combination of papain and hydrogen sulphide than by extracting with either alone. They believe, however, that hydrogen sulphide has an activating effect on β-amylase activity. Myrbäck and Örtenblad (16) consider that hydrogen sulphide has very little stimulating effect and believe that the higher values obtained when hydrogen sulphide is used with papain are the result of activation of the papain by the gas. They stress the importance of using active preparations of papain and claim that with these the total amount of amylase present in the barley can be determined. The authors are of the opinion that the papain used in the present investigation was active. It gave higher values than hydrogen sulphide, whereas Chrzaszcz and Janicki (7) claim that higher values are obtained by the latter method. It seems probable that the papain method extracted all, or at least a very large proportion, of the β-amylase and thus provides a comparative measure of the total β-amylase contents of the samples. The hydrogen sulphide method, though it gave slightly lower results, placed the samples in almost exactly the same order as the papain method, and thus appears to provide an equally valid comparative measure of total β-amylase.

These conclusions are based in part on one other hypothesis that may be questioned, namely, that the papain and hydrogen sulphide methods do not extract any appreciable quantities of α-amylase if this enzyme is present in barley. Chrzaszcz's investigations show that extracts made by these methods have a more rapid action on starch-iodine color than extracts made with water alone. However, the change in color is of the type associated with the action of β-amylase, and the color is not completely destroyed in a comparatively short time as would happen if appreciable quantities of α-amylase were present in the extracts.

It appears that the determination of the free saccharifying activity (Lintner value) of kilned malt provides a comparative measure of the combined saccharifying activities of the free α- and β-amylase present in the samples, although the contribution made by the α-amylase is probably relatively small. Moreover, since data for 16 samples, published by Hills and Bailey (12), give a coefficient of correlation between free saccharifying activity of kilned malt and total saccharifying activity of green malt of 0.974, the present authors consider it probable that the former determination also

provides a fairly good comparative measure of the combined total saccharifying activities of the β-amylase present in barley and of the α-amylase that is elaborated or activated during malting.

Results and Discussion

Varietal Differences

The results of the investigation are summarized in Table I as means, over all stations, for each variety. Owing to the differential effect of environment on varieties, these did not all fall in the same order with respect to any determination at all stations. It was therefore necessary to resort to statistical analyses in order to determine whether the differences between varietal means could be considered significant. The results of the statistical analyses are given in a later section (Table III), but are summarized in the last line of Table I as necessary differences between means required for a 5% level of significance, *i.e.*, for odds of 19 to 1 that a real difference between varieties is operating to spread the means.

TABLE I

Mean saccharifying activities of malt and barley for each variety, in degrees Lintner

Variety	Class	Malt	Barley			
			Total		Free	
		Free, °L.	By papain, °L.	By hydrogen sulphide, °L.	°L.	Per cent of total by papain
A. Olli	6-row, rough awn	153	254	237	54	21
B. Pontiac	6-row, rough awn	131	227	209	96	42
C. Mensury, Ott. 60	6-row, rough awn	129	225	208	94	42
D. O.A.C. 21	6-row, rough awn	127	220	204	95	43
E. Velvet	6-row, smooth awn	124	217	202	97	45
F. Peatland	6-row, rough awn	120	246	231	51	21
G. Hannchen	2-row, rough awn	115	204	180	86	42
H. Victory	2-row, rough awn	103	188	167	82	44
I. Charlottetown 80	2-row, rough awn	100	204	192	46	23
J. Nobarb	6-row, smooth awn	100	197	182	75	38
K. Wisconsin 38	6-row, smooth awn	96	188	172	72	38
L. Regal	6-row, smooth awn	85	163	152	65	40
Necessary difference, 5% level		11	17	16	9	3

A comparison of the necessary differences with the actual differences between the means for the individual varieties leaves no room for doubt that each property measured is a varietal characteristic. The results of the investigation thus provide additional support for Myrbäck's conclusions (14).

The data for the saccharifying activity (Lintner value) of the malts, gienv in the first column of figures in Table I, show that the varieties are divided

into several groups. Olli has by far the highest activity; the rest of the six-rowed rough-awned varieties and the six-rowed smooth-awned variety Velvet form a group with fairly high activity; Hannchen yields an intermediate value; whereas the other two two-rowed varieties and the remaining three six-rowed smooth-awned varieties are low in activity, this being particularly true of Regal.

With respect to the total saccharifying activity of the barley, as measured by either the papain or hydrogen sulphide methods, the varieties fall in much the same rank order. The main discrepancies are caused by Peatland and Charlottetown 80, whose relative positions are improved.

These two varieties, together with Olli, yield anomalous results for free barley saccharifying activity. Whereas the other nine varieties fall in almost the same order with respect to the free saccharifying activities of both malt and barley, Olli, Peatland, and Charlottetown 80 yield very low values for free barley saccharifying activity.

The difference between the two groups of varieties is illustrated further by the data given in the last column, which give free barley activity as percentage of total barley activity (papain). Whereas Olli, Peatland, and Charlottetown 80 yield values of about 22%, the values for all other varieties fall between 38 and 44%.

Relations Between Properties Studied

Between Varietal Means

The more important relations between the various properties studied are illustrated by the scatter diagrams for varietal means shown in Fig. I. Each point is represented by a circle containing a letter from which the variety can be identified by reference to the key given in the first column of Table I.

Fig. 1*A* shows the relation between malt saccharifying activity and total barley saccharifying activity as measured by the papain method, and Fig. 1*B* shows the corresponding relation for the values obtained by the hydrogen sulphide method. The correlation coefficients calculated from the data represented in these two diagrams were 0.904 and 0.868 (the remaining correlation coefficients obtained by the complete analyses of variance and covariance are given in Table IV).

It is apparent that there is a close relation between malt saccharifying activity and total barley activity and that those varieties that give high barley values also tend to give high malt values. This is the conclusion to which Myrbäck comes (14).

Looking at the matter from a different viewpoint, it also seems safe to assume that total β-amylase content, the property measured by the determination of total barley saccharifying activity, is the main factor controlling the comparative saccharifying activities of the malts made from different varieties. The data thus offer further support for the hypothesis advanced by Hills and Bailey (12), namely, that the β-amylase activity of malted grain can be predicted from the β-amylase activity of a papain digest of barley.

The data presented in Fig. 1*A* show, however, that β-amylase content is not the only factor governing the saccharifying activity of the malt though it may be, and probably is, the main one. If it were the only factor we might reasonably expect to find a much closer relation between total barley activity and malt activity, resulting in all the points in Fig. 1*A* falling much more nearly on a straight line.

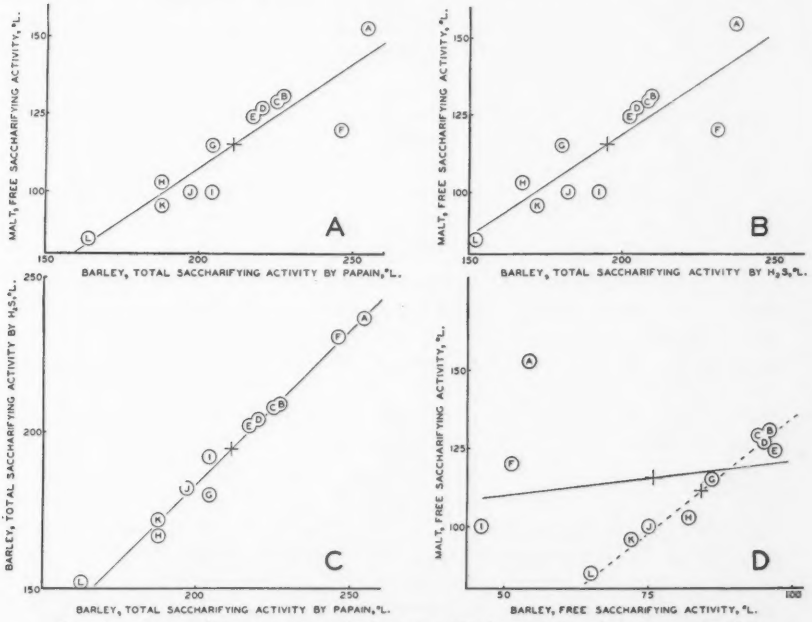

FIG. 1. *Scatter diagrams for varietal means showing relations between saccharifying activities of barleys and malts. The key for varieties is given in the first column of Table I.*

Among other controlling factors, α-amylase, with its contributing saccharifying activity, immediately suggests itself as important, and there are grounds for believing that the varieties differ in α-amylase content. Two possible situations must be considered. The varieties might well differ in α-amylase content, but in such a way that a very close correlation existed between α- and β-amylase contents: those varieties which were high in β-amylase content might also tend to be high in α-amylase content. If such a correlation existed and were close, it would not interfere with the relation between total barley saccharifying activity and malt activity. This situation may exist with respect to eight of the varieties studied. Reference to Fig. 1*A* will show that the points for all varieties except *F*, *I*, *J* and *K* lie almost exactly in a straight line.

The other situation in which the α- and β-amylase contents of varieties vary more or less independently must also be considered. Under these con-

ditions a reduction in the degree of associations between total barley saccharifying activity and malt activity would be expected, since those varieties that were comparatively low in α-amylase content would yield malts with lower activities than would be expected from consideration of comparative β-amylase contents only. In the light of these considerations there are grounds for believing that the varieties Peatland, Charlottetown 80, Nobarb, and Wisconsin 38, particularly the first pair, which are mainly responsible for reducing the correlation between total barley activity and malt activity (*e.g.*, points *F*, *I*, *J*, and *K* in Fig. 1*A*), are comparatively low in α-amylase content.

A difference in the response of varieties to the particular malting conditions used in the present investigation may also be responsible in whole, or in part, for the reduction of the correlation under discussion. Thus it may well be that the standardized set of malting conditions was unfavorable to Nobarb and Wisconsin 38, and particularly unfavorable to Peatland and Charlottetown 80, so that these four varieties failed to develop their potential saccharifying activity as fully as the remaining eight varieties.

Fig 1*C* shows the relation between total barley activity as measured by the papain method and by the hydrogen sulphide method. The correlation coefficient proved to be 0.995. A very close relation is thus demonstrated between the results of the two methods (*cf.* also Table IV), both of which placed the varieties in essentially the same order. It is interesting to note that the points *G*, *H* and *I*, which are furthest from the line, represent the three two-rowed varieties, Hannchen, Victory and Charlottetown 80.

Fig. 1*D* shows the relation between free barley saccharifying activity and malt activity. For nine of the varieties the relation is quite close, the correlation coefficient being 0.971. The other three varieties, Olli (A), Peatland (F) and Charlottetown 80 (I), which have only about 22% of the total barley amylase in free form, spoil the picture entirely and reduce the correlation coefficient to the insignificant value of 0.217. It is thus apparent that the hypothesis advanced by Shellenberger and Bailey (17), namely, that the diastatic activity of malt can be predicted by determining free barley activity, will not hold between all varieties, though data given in Table IV suggest that prediction is possible within individual varieties.

Between Station Means

Mean values for each station, over all varieties, are given in Table II. In order to facilitate comparison of the results of the various determinations, the stations are listed in order of descending values for malt saccharifying activity. Inspection of the data will show that the stations are placed in essentially the same order with respect to each determination. The close relations between malt activity on the one hand and the barley determinations on the other are shown more concisely by the correlation coefficients given in the last line of the table. All these are extremely high. It is thus apparent that most of the environmental factors that control one of the properties measured are also common to the control of the other properties.

The closeness of the relations between malt activity and barley activities is considerably reduced by the performance of the varieties at the first two and the fourth station. Malt values for Beaverlodge and Gilbert Plains are higher, and those for Lacombe are lower than would be expected from consideration of the barley data. It seems probable that these discrepancies are caused mainly by the two factors discussed in connection with between-varieties correlations, namely, differences in the proportions of α- and β-amylase produced under different environmental conditions, and a differential effect of malting conditions on samples from different stations.

TABLE II

MEAN SACCHARIFYING ACTIVITIES OF MALT AND BARLEY FOR EACH STATION, IN DEGREES LINTNER

Station	Malt	Barley			
		Total		Free	
	Free, °L.	By papain, °L.	By hydrogen sulphide, °L.	°L.	Per cent of total by papain
Beaverlodge	150	263	242	102	39
Lacombe	139	281	260	112	40
Ottawa	133	248	229	87	35
Gilbert Plains	133	262	243	88	34
Guelph	122	213	197	76	36
Ste. Anne de la Pocatière	121	218	201	74	34
Brandon	117	215	198	81	38
Lethbridge	116	216	199	83	38
Winnipeg	105	197	181	66	34
Ste. Anne de Bellevue	100	180	165	62	34
Fredericton	85	142	131	50	35
Nappan	63	99	89	33	33
Necessary difference, 5% level	11	17	16	9	3
Coefficient of correlation with malt values	—	.978	.977	.956	—

The last column of figures in Table II gives free barley saccharifying activity as percentage of total barley saccharifying activity by papain. The data show that with increasing total β-amylase content the percentage of free β-amylase remains roughly constant, ranging between 33 and 40%.

Statistical Analyses

The variance of the data for each determination was analyzed into portions due to (i) average differences between varieties; (ii) average differences between stations; and (iii) remainder. The last portion results not only from variations caused by a true interaction between stations and varieties, but also from variations caused by soil heterogeneity within stations, and by sampling and analytical errors. It therefore provides an adequate criterion for testing the significance of differences between station and varietal means.

The mean squares obtained by the analyses of variance are reported in Table III. Since the mean squares resulting from differences in the average performance of the individual varieties are significantly greater than the corresponding remainders, it is apparent that varietal differences exist with respect to each property determined.

TABLE III

ANALYSES OF VARIANCE FOR SACCHARIFYING ACTIVITIES : MEAN SQUARES

Variance due to	Degrees of freedom	Malt	Barley			
			Total		Free	
		Free	By papain	By hydrogen sulphide	Free	Per cent of total by papain
Varieties	11	4372**	7879**	7869**	4047**	1015**
Stations	11	7052**	33056**	28809**	5628**	58**
Remainder	121	166	425	376	120	12

NOTE: *In this and the following table ** denotes that the 1% level, and * that the 5% level of significance is attained.*

TABLE IV

ANALYSES OF VARIANCE AND COVARIANCE FOR SACCHARIFYING ACTIVITIES : CORRELATION COEFFICIENTS

Correlation between	Varieties	Stations	Remainder	Total
Total barley activity by hydrogen sulphide and total barley activity by papain	.995**	.999**	.985**	.997**
Malt activity and total barley activity by papain	.904**	.978**	.816**	.920**
Malt activity and total barley activity by hydrogen sulphide	.868**	.977**	.802**	.913**
Malt activity and free barley activity, *nine varieties only*	.971**	.982**	.781**	.945**
Malt activity and free barley activity, *twelve varieties*	.217	.956**	.649**	.658**
Free barley activity and total barley activity by papain	−.059	.974**	.667**	.652**

Certain pairs of sets of data were also subjected to analyses of variance and covariance. The resulting correlation coefficients are reported in Table IV. It is apparent that the correlation both within and between varieties is very close for total barley saccharifying activity by papain and by hydrogen sulphide, and fairly close for malt activity and total barley activity as measured by either method. For nine of the varieties there is also a correlation both within and between varieties for malt activity and free barley activity, but when all 12 varieties are studied together the inter-varietal correlation drops to an insignificant value. The inter-varietal correlation for

free and total barley activity is also insignificant, but a fairly close correlation apparently exists within varieties.

Applications to Barley Breeding

It appears that the measurement of β-amylase content in barley may prove to be a useful tool for plant breeders that are attempting to select from hybrid material those lines that are satisfactory from both the agronomic and malting viewpoints. The determination can be made quite rapidly (12 per day, per man) and requires only a few grams of grain and no expensive equipment. It should therefore be possible to apply it on a much wider scale, and at an earlier stage in the selections, than is possible with the laboratory malting test.

The possible utility of measurements of total barley saccharifying activity will depend upon whether there is a relation between the β-amylase content and the general malting quality of varieties. This may prove to be true if there are inter-varietal correlations between β-amylase and other hydrolytic enzymes, since it is generally agreed (*cf.* 13, p. 156) that in order to be satisfactory for malting, varieties should be plentifully supplied with amylases, proteases and hemicellulases. Unfortunately the literature on varietal differences in barley appears to contain no adequate body of data bearing on this important point. On the other hand, Hopkins and Krause (13, p. 146) whose opinions must be considered authoritative, think it reasonable to assume that a relation of this sort exists and that varieties that are well supplied with one type of hydrolytic enzyme will also tend to be well supplied with other types. With these considerations in mind, the present authors are inclined to think that the chance of selecting strains of satisfactory malting quality from hybrid lines that are high in β-amylase content will prove to be considerably greater than the chance of selecting such strains from hybrid lines that are low in β-amylase content.

It is apparent that it will be profitable to apply this selection method only to hybrid material resulting from a cross between a parent high in β-amylase content and of generally satisfactory malting quality, and a parent low in β-amylase content. Moreover, owing to the differential effect of environment on varieties, if the hybrid material is grown at one station only, the prevailing environmental conditions may tend to conceal average differences in β-amylase content which might be brought to light if the lines were grown at several stations. In spite of these limitations the selection method appears to be promising, though it is obvious that a good deal of further investigation will be required before its utility can be demonstrated.

Acknowledgments

The authors are pleased to add to the acknowledgments made in Part I (1), their thanks to Mr. W. O. S. Meredith, senior research assistant of the malting laboratory, and to Dr. P. J. Olson, Professor, Department of Plant Science,

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**In Table I of this paper (p. 382) the station names Ste. Anne de Bellevue and Ste. Anne de la Pocatiere should be transposed.*

HYBRIDIZATION OF *TRITICUM* AND *AGROPYRON*

IV. FURTHER CROSSING RESULTS AND STUDIES ON THE F_1 HYBRIDS[1]

By L. P. V. Johnson[2]

Abstract

Results are given of hybridization work involving 27 *Triticum* forms and 18 *Agropyron* species. Only two *Agropyron* species, *A. glaucum* and *A. elongatum*, crossed successfully with *Triticum*. Several hundred F_1 plants have been obtained.

Hybrid seeds varied greatly in size; some were deficient in endosperm, others lacked the embryo. Seed germination and seedling nutrition were materially aided by use of 2 to 5% glucose solutions.

In general, *Agropyron* characters tended to be dominant in inheritance, particularly in crosses involving *A. elongatum*. The dominance relations for important characters are: *Agropyron* dominance in perenniality, vegetative vigor, and extent of mature root; partial *Agropyron* dominance in general morphological type, shattering of rachis, adherence of glumes to seeds, and winter hardiness; intermediate inheritance in texture of mature root, size of seed, rigidity of leaf, and leaf pubescence.

A method of root extraction is described in which plants are grown in special containers that permit reasonably normal root development and greatly facilitate extraction.

F_1 plants of crosses involving *A. glaucum* are completely sterile, while in *A. elongatum* crosses a fair proportion are moderately fertile. Chromosomal associations and relative proportions of functional and non-functional pollen are discussed in relation to fertility.

The more important results from similar work in Russia are outlined and discussed in relation to present results and future prospects of the investigation.

Introduction

Cross-pollination of *Triticum* with *Agropyron*, involving numerous species and varieties of each genus, was carried on extensively during the summers of the years 1935 to 1937. The first year's crossing results and descriptions of greenhouse-grown F_1 plants were reported by Armstrong (1). The present paper gives additional crossing results and F_1 data from both greenhouse and field obtained in 1936–1937, which brings this phase of the work up to date, prior to publication of data on later generations.

Materials and Methods

The *Triticum* materials used for crossing are listed in Table I under the heading "Female parents". Numbers 1 to 10 inclusive are winter varieties of *T. vulgare* (hexaploid, $2n = 42$); 11 and 12 are *T. vulgare*-type, true breeding, biennial wheat and rye hybrids; 13 to 19 are spring varieties of *T. vulgare*; 20 is a spring variety of *T. durum* (tetraploid, $2n = 28$); 21 is a spring form of the tetraploid species, *T. dicoccum*; 22 to 26 are miscellaneous tetraploid species; number 27, *T. monococcum*, is a diploid species ($2n = 14$).

[1] *Manuscript received August 6, 1938.*
Contribution from the Division of Forage Plants, Central Experimental Farm, Ottawa, Canada. This contribution forms a part of a co-operative investigation on the hybridization of Triticum and Agropyron, undertaken by the Dominion Experimental Farms Service and the National Research Council of Canada.

[2] *Formerly Research Assistant, Division of Forage Plants, Central Experimental Farm; now Forest Geneticist, National Research Council of Canada, Ottawa.*

Nearly all these species and varieties are fairly well known in North America. However, certain of the more recent introductions and productions will be described very briefly. Lutescens 0.62, Lutescens 0.329, and *T. timopheevi* were introduced from Russia, the latter being a variety with pubescent stem and leaves reputed to have a remarkable degree of resistance to pests and diseases generally. C.A.N. 1835 (R.L. 1005) is a high quality, rust-resistant variety developed at the Dominion Rust Laboratory from the cross Pentad × Marquis. Canus is a smut-resistant variety recently developed at the University of Alberta.

The readily-crossable *Agropyron* species (Table I) bear Division of Forage Plants numbers as follows: *A. glaucum* 1087, *A. elongatum* 820, 1083, 1300, 1301, 1302, and 1419. *A. glaucum* is hexaploid ($2n = 42$), *A. elongatum* is decaploid ($2n = 70$). These species, described in a previous report (1), are tall, vigorous, polymorphic, perennial bunch grasses having very extensive, fibrous root systems, coarse, harsh foliage, long spikes with shattering rachis, and fairly large seeds with lemma and palea adhering. *A. glaucum* is the shorter, less vigorous, softer in foliage, and more given to spreading by rhizomes. The strains used, especially those of *A. elongatum*, showed considerable variability.

The *Agropyron* species that did not cross successfully with *Triticum* are listed in Table II under the heading "Source of pollen". Division of Forage Plants numbers are given for each species.

Practically all crosses have been made using the *Triticum* parent as female. The reciprocal manner of crossing has the double disadvantage of necessitating emasculation of the stiff-glumed, smaller-flowered *Agropyron* and collection of pollen from the poorer pollen-yielding *Triticum*. It was found that percentage of seed set was much reduced when *Agropyron* was used as the female parent, which is in agreement with the findings of Russian investigators.

The crossing technique used in this work has been described in detail by Johnson and McLennan (5).

Crossing Results

The crossing results for 1936 and 1937 are given in Tables I and II. Table I summarizes the results of crosses involving *A. glaucum* and *A. elongatum*, the *Agropyron* species that cross readily with *Triticum*, while Table II summarizes the results from crossing attempts involving numerous *Agropyron* species that failed to cross successfully with *Triticum*.

In Table I the data from the six strains of *A. elongatum* have been bulked, since it is felt that variation in crossability and morphology within each strain approached the variation between strains.

It should be stated that in the cross Secalotricum × *A. glaucum*, 92 of the 93 seeds recorded in Table I were germless, and that the seeds recorded for *T. monococcum* × *A. glaucum* were also without embryos. Apart from these instances the seeds recorded were reasonably well-developed and potentially germinable.

TABLE I

Triticum-Agropyron CROSSING RESULTS FOR 1936 AND 1937. CROSSES INVOLVING READILY CROSSABLE *Agropyron* SPECIES

Female parents (*Triticum*)	Male parents (*Agropyron*)					
	A. glaucum 1087			*A. elongatum* (six strains)		
	Florets pollinated	Seeds obtained	Per cent success	Florets pollinated	Seeds obtained	Per cent success
1. Dawson's Golden Chaff	868	182	21.0	1,296	39	3.1
2. Minturki	885	32	3.6	425	19	4.5
3. Minhardi	608	99	16.3	–	–	–
4. Lutescens 0.329	1,596	7	0.4	–	–	–
5. Kharkov	665	227	34.1	1,008	14	1.4
6. White Odessa	1,170	173	14.8	–	–	–
7. Yaroslav	–	–	–	1,508	30	2.0
8. Kanred	–	–	–	691	9	1.3
9. Turkey Red	–	–	–	706	17	2.4
10. Crail Fife	–	–	–	1,316	0	0.0
11. Secalotricum	704	93	13.2	–	–	–
12. C.D. 1435	799	92	11.5	738	4	0.5
13. Lutescens 0.62	2,281	58	2.5	531	4	0.8
14. C.A.N. 1835 (R.L. 1005)	1,563	27	1.7	5,394	13	0.2
15. Marquis	902	18	2.0	364	3	0.8
16. Reward	361	26	7.2	–	–	–
17. Canus	664	30	4.5	273	0	0.0
18. C.A.N. 1352	–	–	–	86	2	2.3
19. Chinese	–	–	–	948	38	4.0
20. Mindum	1,228	73	5.9	484	39	8.1
21. Vernal emmer	220	14	6.4	130	0	0.0
22. *T. turgidum* 49	260	4	1.5	284	33	11.6
23. *T. turgidum* 131	348	1	0.3	276	6	2.2
24. *T. polonicum*	60	6	10.0	–	–	–
25. *T. persicum* var. Black Persian	240	20	8.3	94	1	1.1
26. *T. timopheevi*	–	–	–	562	23	4.1
27. *T. monococcum*	83	2	2.4	–	–	–
Total	15,505	1,184	7.6	17,114	294	1.7

Vernal emmer, which gave the highest percentage of crossability obtained with either *Agropyron* species in 1935, was more or less neglected subsequently since an abundance of hybrid seed was secured from the first year's work.

For the sake of simplicity, the *Triticum* material cross-pollinated by non-compatible *Agropyron* species is not itemized in Table II. In general, the results were essentially the same for all *Triticum* forms. An exception that is worthy of note is the disproportionately large number of germless "seeds" produced in crosses involving the wheat-rye hybrid, Secalotricum.

The production of germless seeds and stimulated ovaries from cross pollinations with *A. junceum*, *A. desertorum*, *A. imbricatum*, *A. sibericum*, *A. intermedium*, and *A. dasystachyum* may indicate that these species have relatively closer relations to *Triticum* than do the other non-compatible species of *Agropyron* listed in Table II. Stimulation of ovary development is not, however, considered to be the result of actual fertilization, but rather the result of chemical activation produced by the presence of partially germinated

TABLE II

Triticum-Agropyron CROSSING RESULTS FOR 1936 AND 1937. CROSSES INVOLVING NON-CROSSABLE *Agropyron* SPECIES

Source of pollen	Florets pollinated	Germless "seeds"		Stimulated ovaries	
		No.	%	No.	%
1. *A. junceum* 1086	4274	4	0.09	56	1.31
2. *A. desertorum* 1082	2234	2	0.09	0	0
3. *A. imbricatum* 995	1076	2	0.19	0	0
4. *A. sibericum* 1159, 1163, 1164, 1165	2890	0	0	2	0.07
5. *A. intermedium* 1085	3806	5	0.13	11	0.29
6. *A. dasystachyum* 931	1036	0	0	3	0.29
7. *A. cristatum* 653, 1081, 1158	3355	0	0	0	0
8. *A. cristatum* 655 (var. Fairway)	1549	0	0	0	0
9. *A. pauciflorum* 1267	640	0	0	0	0
10. *A. obtusiusculum* 1077	145	0	0	0	0
11. *A. Griffithsii* 1443	719	0	0	0	0
12. *A. Smithii* 73	530	0	0	0	0
13. *A. repens* 1076	40	0	0	0	0
14. *A. spicatum* 1060	210	0	0	0	0
15. *A. caninum* 1080	128	0	0	0	0
16. *A. Richardsonii* 932	40	0	0	0	0
Total	22,672	13	0.06	72	0.32

Agropyron pollen on the *Triticum* stigma. This interpretation suggests the possibility of relatively closer phylogenetic affinities of the species in question. Germless "seeds", however, are probably the result of partial fertilization, since there is a definite degree of endospermic development. It is believed that fertilization proceeded, in the latter, to the point where one of the sperm nuclei united with the secondary (or fusion) nucleus of the embryo sac to activate endospermic development, but that it did not proceed to the fusion of the second sperm nucleus with the egg nucleus (which would have initiated embryonic development). This presumes a much closer relation between species involved than mere stimulation of the ovary by chemical transmission.

In *T. persicum* var. Black Persian pollinated by *A. junceum*, one seed was produced which germinated and gave rise to a mature plant that was proved cytologically to be hybrid. Unfortunately, this plant died in transplanting.

Description of Hybrid Seeds

The effects of cross-pollination, in different and in the same parental combinations, ranged from slight to pronounced stimulation of the ovary, through intermediate gradations of endospermic without embryonic development, and varying degrees of endospermic with embryonic development, to the condition of a seed well developed in all respects (Plate I). Only seeds potentially germinable and with a reasonable amount of endosperm are considered in this section.

Hybrid seeds from different crosses exhibited great variation in shape and size which was closely correlated to the shape and size of seed in the respective

female (*Triticum*) parents. This is, of course, to be expected since *(a)* the 2*n* condition of maternal nuclear contribution to endospermic development would tend to emphasize maternal characteristics, *(b)* the seed coats are wholly maternal tissue and *(c)* the space conditions determined by the flowering glumes would tend to mold the shape and size of any seed developed therein.

A further variation related to hybrid seeds was the pronounced difference in germinability (or, more strictly speaking, the capacity for continued seedling development after the initiation of germination), the range being from 96.3% (Mindum × *A. glaucum*) to 11.9% (C.A.N. 1835 × *A. glaucum*). Such variation in germinability showed a close, direct correlation to seed weight. This correlation may be explained by assuming that the endospermic reserves in light seeds were insufficient to carry seedling nutrition to the point where rootlet absorption and photosynthesis take over the nutrition of the plant.

Cultural Methods

Germination of Hybrid Seed and Nutrition of Seedlings

The development of hybrid seedlings from seeds sown on or in sand and watered with tap water or a complete nutrient solution was often very poor, especially when seeds were markedly deficient in endosperm. Examination of such sowings revealed very frequent occurrence of seeds that had ceased development at an early stage. These observations led to experimentation designed to provide information on the nutrition of embryos in seeds deficient in endosperm. Since hybrid seeds were considered too valuable to subject to experimental risks, substitute material was prepared by excising (with small amount of endosperm retained) *Triticum* embryos (C.A.N. 1835), and by cutting the embryo ends (barely including the embryo) from seeds of *Agropyron elongatum* D.F.P. No. 1083.

Approximately 1000 gm. of sand was placed in each of 48 seven-inch, low-type, unglazed pots which were then allowed to stand in a shallow tray of water until thoroughly moistened. Twenty-five wheat embryos were sown to a depth of exactly $\frac{3}{8}$ in. in each of 24 pots, and the remaining 24 pots were similarly sown with clipped seeds of *Agropyron*. Four pots, two replications of each type of seed, were used to test each of the solutions used (Table III). Nowosad's *F* is a complete inorganic nutrient solution recommended for growing grasses and cereals in sand culture.

In order to observe directly the germinative behavior in the various solutions, 10 seeds from each material were sown on a blotter disc in a Petri dish, one dish to each of the 12 solutions. The discs were first moistened with tap water, then wetted with an excess of the solution.

After seeding, 24 cc. of the proper solution was added to each pot. The pots were flushed with 15 to 20 cc. of tap water about every second day (every day if evaporation was great). Six cc. of solution per pot was added by pipette each day for eight days, and thereafter on alternate days. The

guiding principle was to maintain approximately the original concentration of the solution, compensating for evaporation by the addition of water, for percolation and utilization by the addition of solution. Under the conditions of the experiment, efficiency in maintaining these conditions was entirely a matter of personal observation and judgment. The work was carried out under cool, damp, dull conditions in early winter. The experimental conditions could be improved greatly by the use of glazed pots and a humidity chamber.

Seedling emergence was general by the seventh day after sowing. The final seedling counts recorded in Table III were made on the fifteenth day.

TABLE III

DATA DEMONSTRATING THE EFFECTIVE NUTRITION PROVIDED TO EXCISED EMBRYOS OF *Triticum* AND *Agropyron* BY VARIOUS SOLUTIONS

Solution	No. of seedlings emerged, pots					No. of seeds germinated, Petri dishes			
	Triticum		*Agropyron*		Total out of 100	*Triticum*	*Agropyron*	Total out of 20	%
	1	2	1	2					
Tap water	0	1	5	4	10	5	8	13	65
Nowosad's F	0	0	3	4	7	4	5	9	45
Glucose 0.5%	3	1	9	11	24	6	7	13	65
Glucose 1.0%	0	2	8	13	23	7	8	15	75
Glucose 2.0%	3	4	13	13	33	7	8	15	75
Glucose 3.0%	10	9	13	19	51	7	7	14	70
Glucose 5.0%	8	13	18	17	56	8	8	16	80
Maltose 0.5%	0	0	10	10	20	8	7	15	75
Maltose 1.0%	1	0	9	6	16	7	7	14	70
Maltose 2.0%	1	1	5	4	11	7	9	16	80
Maltose 3.0%	0	0	6	4	10	6	10	16	80
Maltose 5.0%	0	1	5	6	12	5	8	13	65

The emergence data in Table III demonstrate that glucose solutions in all concentrations give a marked increase over the control, while the germination (direct observation) data indicate only a slight increase from these solutions. This may be explained by assuming that about 65% of the embryos were capable of germination but that sugar nutrient was required to enable the seedlings to emerge through $\frac{3}{8}$ in. of sand. Maltose solutions appear to have stimulated germination, but were indifferent in emergence tests. The latter result may have been due to the definite crusting of the sand at the surface with maltose solutions of 1% or over. The complete nutrient solution (Nowosad's *F*) was detrimental to germination. This type of nutrition is, apparently, effective only after rootlet absorption and photosynthetic activity have become established.

At this stage of the experiment the pots were thoroughly flushed and the plants grown for several weeks with complete nutrient solutions (Nowosad's *F* and others).

Two per cent glucose solution was applied (flushed alternately with water) to several hundred hybrid seeds sown in sand. As a control, one of two trays

of comparable material was treated with glucose, while the other received water only. Emergence from the tray treated with glucose was 128 seedlings from 148 seeds (86.5%), while only 81 seedlings were obtained from 125 seeds (64.8%) in the tray receiving water only.

Growing of F_1 Plants

Since the F_1 plants were perennial, and would therefore normally flower for the first time in the second year of their existence, and since it was desirable to induce flowering in the greenhouse during the first winter, special treatment was required. The hybrid seeds were collected from cross-pollinated *Triticum* plants in August and sown in sand in greenhouse flats in late September. About two weeks later the seedlings were transplanted to soil in 2½-in. pots in which they remained, under the coolest possible greenhouse conditions, for several weeks. During this time the leaves were cut back successively and advantage taken of opportunities to place the plants under cool outdoor conditions and gradually to subject them to mild freezing. This treatment simulated the winter conditions which biennial and perennial plants normally require before flowering. About the middle of November the plants were placed under optimum growing conditions for about a week and then transplanted to 5-in. pots, where they remained until maturity. Early in January the period of artificial illumination was increased so that total daily illumination was 17 to 18 hr. Flowering commenced early in February and, in the fertile plants, seed was matured by late March. The plants were then cut back and superior individuals cloned and repotted. Plants were placed out of doors early in May and transplanted to the field about two weeks later.

Description of F_1 Plants

Seedling Characters

Observations on number of rootlets on germinated seeds of parents and hybrids may be summarized as follows: spring wheats usually have five or six rootlets, maximum nine; winter wheats tend to have a lesser number; *A. glaucum* usually has two or three rootlets, *A. elongatum* one or two. These parental characteristics are reflected in hybrid seedlings. Hybrids between spring wheats and *A. glaucum* tend to average three or more rootlets, while hybrids of spring wheats and *A. elongatum* tend to average less than three; when winter wheats are involved, these averages tend to be less. In rootlet number the *Agropyron* parents exert a dominant heritable influence which is, however, not quite complete, especially in *A. elongatum*. Most of these points substantiate the data given in Table VI of Armstrong's report (1).

Color of the coleoptile was studied in parental and hybrid material. The coleoptile of *A. glaucum* and *A. elongatum* is purplish, while in most wheats it is green. In the hybrids, coleoptile color tended to be like that of the *Agropyron* parents.

Observations on juvenile posture (angle between leaves and the ground), made at about the five-leaves stage, reveal a marked tendency for the semiprostrate condition of the *Agropyron* parent to be transmitted to the hybrid.

It is hoped that correlations between seedling characters and important mature-plant characters in segregating generations may prove useful in the selection of superior types at the seedling stage.

General Upper-plant Characters

The previous report by Armstrong (1) gave general and detailed descriptions of the F_1 hybrids and parental types grown in the greenhouse. The present descriptions are more detailed and are based on more extensive observations on both greenhouse and field-grown plants. They may be considered as being supplementary to the previous descriptions.

During the late winter of 1936, detailed observations were made on greenhouse material, both hybrid and parental, at the time of flowering or shortly thereafter. The data from these observations are given in summarized form in Tables IV and V. In summarizing, the data compiled from each of several plants studied in a given material were arithmetically or otherwise averaged for each separate character. In this way a single term was obtained which indicates the average or typical expression of the character in question.

Table IV presents summarized descriptions of spike and culm characters, while Table V deals similarly with leaf characters.

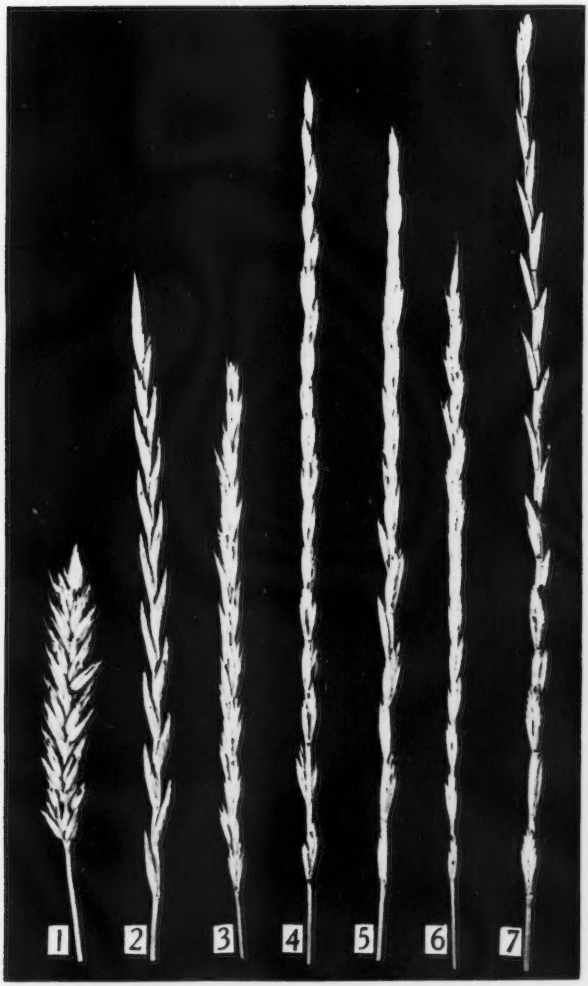
The data in Tables IV and V will be discussed in connection with Table VII, which serves further to summarize these data as they relate generally to the phenomena of dominance, transgressive expression and hybrid vigor.

After growing to maturity in the greenhouse, the hybrid materials (upon which data in Tables IV and V are based) were cloned and transplanted to the field in the spring of 1936. Field observations made throughout the summer are summarized in Table VI.

In the field the hybrid material, with one exception, grew very vigorously. The exception was the Mindum × *A. glaucum* material, in which about 90% of the plants gradually died or formed a few, weakly erect or decumbent stems. In the case of vigorous hybrids of this cross, all individuals of the clone were vigorous, and the same clonal uniformity was true with respect to non-vigorous hybrids. Beyond all reasonable doubt, therefore, the developmental response was genetic rather than purely environmental.

In the first year's field growth, the occurrence of tufted plants was particularly prevalent in Kharkov × *A. glaucum*, C.A.N. 1835 × *A. glaucum*, and Vernal emmer × *A. elongatum* materials. This response appeared to be related in most crosses to perenniality, while in others, notably Kharkov × *A. glaucum*, it appeared to be due to developmental disturbances probably related to hybridity. In every instance the response was uniform among the individuals of the clone, indicating a genetic rather than a purely environmental basis.

The susceptibility to ergot found in *A. glaucum* crosses is, in all probability, inherited from *A. glaucum*, which is fairly susceptible. The degree of susceptibility shown by *A. glaucum* hybrids involving Kharkov, Lutescens 0.62, and Vernal emmer is, however, considerably greater than has been observed



Mature spikes of: 1. *Triticum vulgare var. Lutescens 0.62.* 2. *Lutescens 0.62 × A. glaucum.* 3. *C.A.N. 1835 (T. vulgare, bearded) × A. glaucum.* 4. *Lutescens 0.62 × A. elongatum.* 5. *C.A.N. 1835 × A. elongatum.* 6. *A. glaucum.* 7. *A. elongatum.*

TABLE IV

SUMMARIZED DETAILED DESCRIPTIONS OF SPIKE AND CULM CHARACTERS TYPIFYING CERTAIN PARENTS AND HYBRIDS GROWN IN THE GREENHOUSE

Material	No. of plants	Spike							Culm				
		Density index	No. of spikelets	No. of florets	Awning	Secondary glume			Plant height, in.	No. of nodes	Pubesc. of node	Diam. culm, mm.	Diam. neck, mm.
						Shoulder	Beak	Keel					
A. glaucum	5	89.0	18.8	3–4	1	N	M, M–A	M	50.6	4.0	0	1.79	1.10
A. elongatum	10	139.8	14.24	5–7	0	N–M	0	Wk–M	54.9	3.4	0	2.21	1.37
Lutescens 0.62	5	58	16.2	4	0–1	W	S, B	St	38.8	3.0	+	3.19	1.79
Lutescens 0.62 × *A. glaucum*	12	67.6	19.8	3–4	0–1	N	S, A	St	66.0	4.3	0	2.41	1.41
Lutescens 0.62 × *A. elongatum*	10	100.5	15.7	4–6	0	M	0–S, A	Wk–M	63.7	3.5	0	2.35	1.39
C.A.N. 1835	6	49.3	23.9	4–5	3	M	L, A	M	53.3	4.2	+	3.64	2.48
C.A.N. 1835 × *A. glaucum*	8	68.4	17.7	3–4	0–1	N	S–M, A	St	50.5	4.3	0	2.16	1.27
C.A.N. 1835 × *A. elongatum*	10	118.9	13.6	4–6	0	M	0	Wk	59.1	3.2	0	2.29	1.34
Kharkov	5	47.1	26.0	4	3	W	L, A	St	54.8	4.8	Tr	3.30	2.00
Kharkov × *A. glaucum*	11	72.0	20.1	3–5	0–2	N–M	S–L, A	St	53.5	4.4	0	2.47	1 52
Kharkov × *A. elongatum*	17	108.0	14.1	4–6	0–Tr	M–W	0–S, A	M	58.6	3.4	0	2.26	1.28
Mindum	5	34.2	30.6	3–4	3	N	L, A	St	55.2	4.7	0	4.62	1.26
Mindum × *A. glaucum*	10	59.5	17.9	3–4	1–2	N	S–M, A	St	59.1	4.3	0	2.13	1.07
Mindum × *A. elorgatum*	4	101.0	11.0	3–4	0–Tr	N	0–Tr	Wk–M	54.0	3.9	0	1.69	0.98
Vernal emmer	5	30.3	24.2	3–4	3	N	S, B–A	St	56.4	4.3	+	2.71	1.29
Vernal emmer × *A. glaucum*	10	54.0	22.0	3	1–2	N	S, A	St	59.8	4.2	0	2.26	1.17
Vernal emmer × *A. elongatum*	10	95.8	11.2	4–6	0	M	0–S, A	M	59.6	4.4	0	1.66	0.97

EXPLANATION: Spike density index refers to the distance in mm. along the spike occupied by ten typical spikelets, thus the larger the index the smaller the density. Under awning, 0 denotes awnless, 1 short awns, 2 medium awns, etc. The secondary glume is the outer glume that originates slightly above the opposing outer glume; it is very constant in expressing varietal differences. Under secondary glume beak, the symbol preceding the comma refers to length of beak, while the symbol following the comma refers to sharpness of beak. Culms were measured by micrometer about 2 in. above second topmost node, necks were similarly measured about an inch below spike.

SYMBOLS: 0—absent; Tr—trace; +—present; N—narrow; M—medium; A—acute; B—blunt; S—short; L—long; Wk—weak; St—strong.

TABLE V

SUMMARIZED DETAILED DESCRIPTIONS OF LEAF CHARACTERS TYPIFYING CERTAIN PARENTS AND HYBRIDS GROWN IN THE GREENHOUSE

Material	No. of plants	Leaf											
		Length, cm.	Width, mm.	Pubescence			Venation			Marginal barbing	Rigidity	Auricle color	Ligule length
				Dorsal	Ventral	Marginal	No.	Barbing					
								Dorsal	Ventral				
A. glaucum	5	17.6	7.5	0–S	M–L	0–¾L	7.4	0–M	St	St	M	Wh	S–M
A. elongatum	10	17.0	4.9	0	0–M	0–BM	5	Wk	St	M–St	St	Wh	S–M
Lutescens 0.62	5	26.4	14.4	S	S	BS	10.6	0	+	M	Wk	Wh	M
Lutescens 0.62 × *A. glaucum*	12	20.2	9.9	S	M	CS–¾M	8.2	Wk	+	St	Wk–M	Wh	S–M
Lutescens 0.62 × *A. elongatum*	10	19.3	7.5	0–S	S–M	0–BS	7.5	Wk	M	M–St	M	Wh	S
C.A.N. 1835	6	31.4	17.7	S	S	CS	11.8	Wk	+	M	Wk–M	Wh	M
C.A.N. 1835 × *A. glaucum*	8	13.0	9.5	0–S	0–S	0–CM	8.2	0	+	Wk–M	Wk–M	Wh	S–M
C.A.N. 1835 × *A. elongatum*	10	21.0	7.3	0–S	S–M	0–BS	7.2	0–St	Wk–St	M–St	M–St	Wh	S
Kharkov	5	18.1	12.6	0	0	CM	9.6	+	M	Wk	M	P	L
Kharkov × *A. glaucum*	11	16.9	10.9	0	0–S	0–BS	8.4	0–M	+	Wk–M	M	P	S
Kharkov × *A. elongatum*	17	21.1	7.2	0	0–M	0–½M	7.0	0	St	St	M–St	P	M
Mindum	5	18.5	12.5	0	0	CS	9.2	Wk	+	Wk	Wk	Wh	M–L
Mindum × *A. glaucum*	10	16.1	8.2	0	0–S	0–BS	7.7	0–Wk	+	M	Wk–M	Wh	M
Mindum × *A. elongatum*	4	15.3	4.7	0	0–S	0–BS	6.9	Wk	St	M	M–St	Wh	S
Vernal emmer	5	22.2	12.0	Tr	S	BS	9.3	0	0	Wk	Wk	Wh	L
Vernal emmer × *A. glaucum*	10	17.6	9.7	0–Tr	S–L	CS–¾M	7.9	0	0	M–St	Wk–M	Wh	M
Vernal emmer × *A. elongatum*	10	16.6	5.7	0–S	S–M	0–CS	7.0	Wk	M	M	M	Wh	S–M

EXPLANATION: Highest typical leaf used, usually topmost, occasionally second topmost. Under pubescence marginal, combinations of symbols are to be read as follows: 0–¾ L denotes a range from absence to long hairs for three-quarters of length of leaf, BS denotes short hairs at base of leaf, MC denotes medium length hairs at colar of leaf, etc.

SYMBOLS: 0—absent; Tr—trace; +—present; S—short; M—medium; L—long; ½, ¾—portion of leaf affected; B—base; C—colar; St—strong; Wk—weak; Wh—white; P—purple.

TABLE VI

SUMMARIZED GENERAL DESCRIPTIONS OF CERTAIN HYBRIDS GROWN IN THE FIELD

Cross	No. of plants	Days to heading	Posture	Relative height	Relative no. of culms	Leafiness	Stem (solid or hollow)	Reaction to		Forage quality	Remarks
								Ergot	Leaf rust		
Mindum × *A. glaucum*	52	41-98, 57	1-4, 2	1-3, 2	1-3, 1	1-2, 1	S-H, S-H	0-2, 1	1 2, 1	Poor-fair; short, rather harsh leaves.	4 plants of fair-good forage quality.
Kharkov × *A. glaucum*	35	45-96, 61	2-3, 2	1-3, 3	1-4, 3	1-3, 2	S-H, H	1-4, 3	0-3, 2	Poor-fair; medium long, rather harsh leaves.	2 plants of fair-good, many tufts.
Lutescens 0.62 × *A. glaucum*	21	45-54, 49	2-3, 3	2-3, 3	3-4, 4	3 3	S-H, S	1-4, 3	1-2, 1	Poor-fair; long, rather harsh leaves.	Very uniform.
C.A.N. 1835 × *A. glaucum*	7	45-60, 52	2-3, 2	1-3, 2	1-4, 3	1-3, 2	S-H, S	1-2, 1	1-2, 1	Poor-fair; short, rather harsh leaves.	Many tufts.
Vernal emmer × *A. glaucum*	53	45-84, 57	2-4, 3	2-3, 3	3-4, 4	3-4, 4	S-H, S	0-3, 2	0-2, 1	Poor-fair; medium long, rather harsh leaves.	
Mindum × *A. elongatum*	6	56-84, 71	3-4, 4	2-3, 3	2-4, 3	3, 3	S-H, S-H	0	1, 1	Poor-fair; narrow, rather harsh leaves.	Considerable variation.
Kharkov × *A. elongatum*	38	41-68, 52	2-3, 2	2-4, 3	3-4, 3	2-3, 2	S-H, S-H	0	1-2, 1	Poor; stiff, rather harsh leaves.	
Lutescens 0.62 × *A. elongatum.*	16	43-59, 47	2-3, 2	2-4, 3	2-4, 4	2-3, 2	S-H, S-H	0	1-2, 1	Poor; stiff, rather harsh leaves.	
C.A.N. 1835 × *A. elongatum*	13	45-63, 51	2-3, 2	3, 3	3-4, 4	2-3, 2	S-H, S-H	0	1-2, 1	Poor; stiff, rather harsh leaves.	
Vernal emmer × *A. elongatum.*	51	55-102, 75	3-4, 3	3-4, 3	3-4, 4	2-3, 2	S-H, S-H	0	0-2, 1	Poor; stiff, rather harsh leaves.	Many tufts.

EXPLANATION: Numerical increase denotes quantitative increase in character expression: thus, 1 denotes short, 2 medium short, 4 tall, etc. In the case of posture, 1 denotes prostrate, 4 denotes erect. In each column the data preceding the comma refers to the range of expression, while the data following the comma refers to the average or typical expression.

in *A. glaucum*. The wheats, parental in these instances, have not been observed to be particularly susceptible to ergot. The characteristic ergot immunity of *A. elongatum* is reflected in its hybrids.

Quality of forage varied greatly with age and position of the leaves. New leaves, and tufted leaves at the base of the plant, were relatively much softer than older or upper leaves. In general, the *A. glaucum* hybrids have the softer foliage, the best in this respect being Mindum × *A. glaucum*. Unfortunately, this hybrid is usually non-vigorous. Among *A. elongatum* crosses, Mindum also gave the softest foliage, combined with excellent vigor. Among the *Triticum* parents, Mindum has noticeably the softest leaves.

The vegetative period in all hybrids, but particularly in those of *A. elongatum*, is prolonged into late fall. The degree of expression in this regard exceeds the limits of either the *Triticum* or *Agropyron* parents (transgressive expression) and appears, with exception of *A. glaucum* crosses with Mindum and C.A.N. 1835, to be related to hybrid vigor.

The winter hardiness of the hybrids was severely tested during the winter of 1936–37 when the amount of winter killing among perennial crops was extremely high. In general, it may be said that the *Triticum-Agropyron* hybrids survived about as well as native *Agropyrons*. Both *A. glaucum* and *A. elongatum* hybrids involving Kharkov, the only winter-wheat parent at the time, survived much better than any other. This observation led to the emphasizing of winter wheats as *Triticum* parents in subsequent crossing. The winter of 1937–1938 was not sufficiently severe to bring out differential reactions to freezing.

Hybrids of *A. glaucum* have a moderate tendency to spread by short rhizomes, a characteristic inherited from *A. glaucum*. The *A. elongatum* hybrids, like their *Agropyron* parent, have not rhizomes, but by profuse tillering attain large, bushy proportions at maturity.

Perenniality appears to be completely dominant in all F_1 hybrids. It remains to be seen, however, whether the hybrids will live as long as their *Agropyron* parents.

Leaf Characters Related to Drought Resistance and Palatability

Since drought resistance and forage palatability are both primary considerations in the practical objectives of the work, leaf characters related to these qualities are very important. Preliminary studies of a comparative nature have been made on cross sections and stomatal surfaces of the leaves of the F_1 plants and parents of the more important crosses (those dealt with in Tables IV and V).

Leaf material for cross sections was collected from field-grown plants immediately after flowering, central portions of typical upper leaves being taken. A 6% solution of formalin proved to be superior as a killing and fixing agent to Navashin's or alcoholic agents, since it caused less hardening and facilitated cutting, which was done with a hand microtome using pith imbedding. Sections were cut at approximately 50 μ, fixed on slides with

Szombathy's agent (4), stained by modifications of Flemming's safranin, gentian-violet and orange G, or Chamberlain's safranin and light green schedules (4) and mounted in balsam. Differential staining was good with either schedule, with preference for the triple stain. After preliminary examinations of stained sections, general comparative observations were made on freshly cut, unstained temporary mounts.

Material (either fixed or fresh) for stomatal work was prepared by placing downward the epidermis to be studied and scraping away all other tissues with a razor blade. Excellent differential staining was obtained with safranin (1 gm. in 150 cc. of 50% ethyl alcohol).

Observations on cross sections of the leaves of parents and hybrids indicated a very similar relation between parents and hybrids in all crosses. This permits a general discussion of observations with only occasional reference to specific materials.

In cross section, the upper surface of the leaf of *A. elongatum* appears as a continuous series of alternately high and low, steep-sided, round-topped undulations (ridges and furrows). Bulliform (motor) cells occur at the base of the ridges, and stomata occur in a staggered row on either side of the ridge. Girder tissue of thick-walled, sclerenchymatous cells extends on either side of the vascular bundle, with increasing width, to the epidermis. The lower surface of the leaf is non-ridged with stomata between girder outcroppings. These general characters are transmitted with a few minor differences to *Triticum* × *A. elongatum* hybrids. Influence of *Triticum*, which is only slightly ridged, is expressed in the hybrids as a marked flattening of the tops of the ridges. While the amount of girder tissue in the hybrids is about the same as in *A. elongatum*, the cell walls of the hybrid tissue are much thinner, being intermediate between the parents. This probably accounts for the intermediacy of leaf rigidity in the hybrids (Tables V and VII).

Thickness of cuticle of the upper epidermis is much greater in *A. elongatum* than in *Triticum*, and this character is to a considerable degree transmitted to the hybrids. The cuticle of the lower epidermis is thick in *A. elongatum* and moderately thick in *Triticum*. This lack of clear-cut difference makes classification of the hybrids difficult, but they are believed to have a slightly thicker cuticle than the *Triticum* parents.

The differences between *A. glaucum* and *Triticum* for most of the characters discussed above are not very pronounced. While the genetic transmission of characters to the hybrids is not easily observed, it is considered to be essentially the same as for *A. elongatum* hybrids. The amount of girder tissue in hybrids reflects the greater amount present in the *A. glaucum* parent, but cell-wall thickness in the hybrids is considerably thinner owing to a *Triticum* influence. The flattening of the top of ridges in the hybrids, mentioned above, is also noticeable in *A. glaucum* hybrids.

A. elongatum has a pronounced, continuous, parenchymatous sheath surrounding the vascular bundle, while in *A. glaucum* and the *Triticum* parents this sheath tends to be less pronounced and somewhat discontinuous.

Triticum × *A. elongatum* hybrids appear to be identical with their *Agropyron* parent in this character.

Stomatal counts proved to be so variable that significant differences could not be established.

A survey of some of the literature dealing with xeromorphic characters reveals a wide difference in the emphasis placed by various workers upon the importance of these characters to drought resistance. In the present paper this question is left entirely open. There are several so-called xeromorphic characters represented in the leaves of the *Agropyron* parents, especially *A. elongatum*. These may be listed as follows: ridging of leaves, bulliform cells, and presence of stomata in leaf furrows, considered by some to be jointly related to drought resistance in connection with leaf rolling; sclerenchymatous tissues and parenchymatous sheaths surrounding vascular bundles as a possible protection against excessive water loss through spongy tissues; relatively large proportions of sclerenchyma as compared with parenchyma; and heavy cuticular development. These characters tend to be transmitted to the hybrids.

It is important to note, however, that many of these characters, considered favorable from the point of view of xerophytism, are extremely unfavorable from the point of view of forage palatability. It may be necessary, therefore, in subsequent breeding work, to effect a compromise between xeromorphism and forage quality.

More complete histological data, discussed from the viewpoint of xerophytism, are given by McLennan (6).

Root Characters

Comparative studies have been begun on the root systems of parents and hybrids in order to provide practical information that, together with that derived from histological studies of leaves, might contribute toward a better understanding of the morphological bases of drought resistance.

The great obstacle in root studies is the extremely laborious task of root extraction. A special container in which the roots are grown was devised which affords an approach to the ideal of maximum reduction in time and labor of extraction with minimum reduction in quality of root. In its unassembled condition the container consists of two pieces of galvanized sheet metal each bent to form a right-angular, V-shaped trough. Along one of the straight edges of each section, the metal is bent to make a groove about an inch deep which will admit the unbent straight edge of the other. In assembling, the two sections are held in place by small bolts inserted at four points along the connection. The assembled container is a box-like object 36 in. high, 16 in. square and open at both ends.

Forty-nine containers were placed in seven equal rows, slightly sunken in cultivated soil and banked with soil to the top edges of the outermost containers. In filling the containers, an effort was made to simulate natural soil horizons and care was taken to pack the soil thoroughly and evenly at

the time of filling. If possible, the soil should be prepared one year in advance, particularly in dealing with annual plants. Plants are started from seed or from very small, uninjured seedlings.

When a given root system is to be extracted, the container with plant and soil intact is removed to a tank of water, laid on its side and soaked overnight, after which the upper section of the container is removed and, beginning at the root end, extraction is made by the Pavlychenko spray method (7). The container proved to be near the maximum size that could be handled without special means for lifting and moving.

This method was quite satisfactory for extensive perennial roots, especially if two or more years old. Difficulty with breaking of the soil block was experienced in moving containers in which annuals were growing in soil which had been filled in only a few months previously. This difficulty was largely overcome by permitting soil to settle in the container one year before use.

The root systems obtained by this method are not complete, nor can they be considered strictly typical of field-grown roots. Nevertheless, they are considered to be sufficiently similar to normal roots in type and extent to permit evaluation for genetic and breeding purposes.

During the summer of 1937 some 20 roots were extracted, of which 15 were preserved in 4% aqueous formalin solution for later detailed studies. These roots are as follows:

C.A.N. 1835	
Kharkov	(two roots)
A. elongatum 820	(one second-year root)
A. elongatum 1083	(two second-year roots)
A. glaucum	(two second-year roots)
Kharkov × *A. elongatum* 1083, F_1	(two second-year roots)
Vernal emmer × *A. elongatum* 820, F_1	(two second-year roots)
Vernal emmer × *A. glaucum*, F_1	(two second-year roots)
Lutescens 0.62 × *A. elongatum* 1083, F_1	(one second-year root)

Preliminary observations on roots of the *Agropyron* parents reveal very extensive systems with large numbers of main branches which tend to be coarse, especially in *A. elongatum*. The wheat parents, especially spring wheats, have smaller root systems with branches of much finer texture. The root systems in the F_1 hybrids are fully as extensive as those of the *Agropyron* parents and show a pronounced tendency toward the fine-textured branches of the *Triticum* parents (Plate II). This is considered to be a very favorable combination of parental characteristics.

It is questionable whether the occurrence of very extensive root systems in F_1 plants is due to dominance of the *Agropyron*-type root or to hybrid vigor. Observations in F_2 roots should provide an answer to this question.

These preliminary observations on F_1 hybrid root systems indicate the probable occurrence of a preponderance of superior roots among hybrid segregates. There is no reason at present, therefore, to doubt the possibility

of selecting hybrid plants having root systems that will provide resistance to drought and that will have good soil-binding properties.

Summary of Descriptions of F_1 Plants

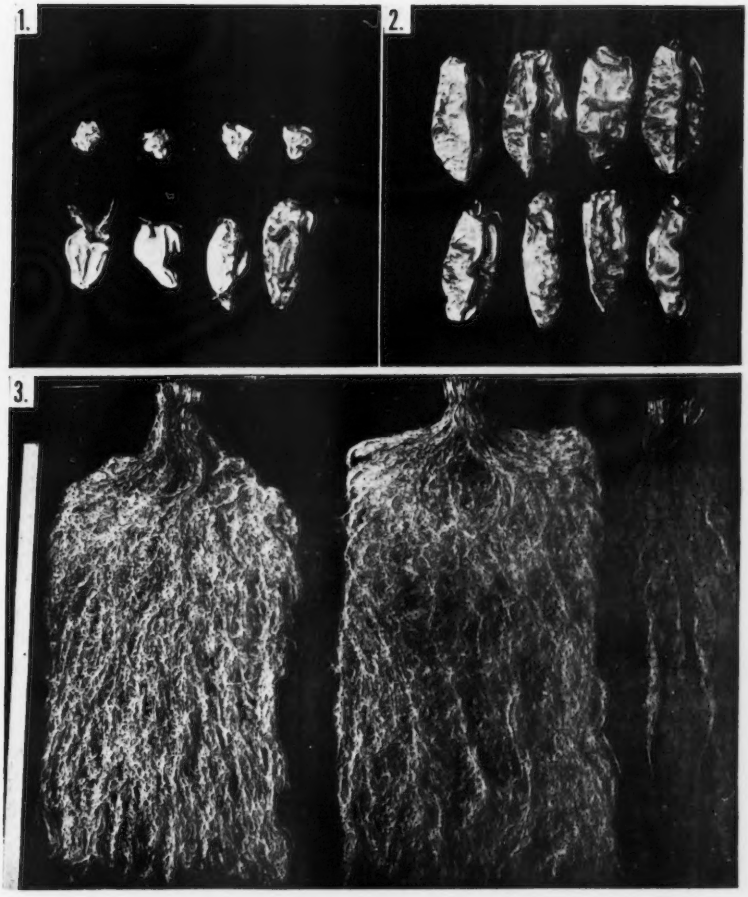
To facilitate general summarization the descriptive data compiled from the original, and more thoroughly studied, F_1 hybrids have been brought together in Table VII in which they are examined as a whole, on the basis of relative genetic dominance, transgressive expression, and hybrid vigor (compare Table VII of Armstrong's report (1)).

The symbols in Table VII are used as follows: *A* and *T* denote apparently complete dominance of the *Agropyron* and *Triticum* conditions, respectively; *I*, *IA* and *IT* denote intermediate, intermediate-*Agropyron* and intermediate-*Triticum* conditions, respectively; *X*, *XA* and *XT* denote indeterminate transgressive expression, and transgressive expressions transcending *Agropyron* and *Triticum* parents, respectively. The term "transgressive expression" denotes a degree of expression that goes beyond the limits observed in the parents; the degree of expression may be lower than the low-degree parent, higher than the high-degree parent, or it may be indeterminate, *i.e.*, not definitely related to the type of expression found in either parent. The dashes indicate lack of character differential in the parents, lack of expression in hybrids, or lack of data.

Certain characters included in Table VII but not discussed previously, and concerning which there may be some vagueness, will be discussed briefly. The term "life tenure" refers to the condition of being annual, perennial, etc. The perennial *Agropyron* condition appears to be completely dominant. The "heading-to-flowering" period (time from spike emergence to anthesis) differs widely in the parents, being for *Triticum* usually less than a week, for *Agropyron* two or more weeks, and for the hybrids the same as for *Agropyron* (or slightly less). "Posture" refers to the condition of being erect, prostrate, etc. In juvenile posture (five-leaves, seedling stage) *Agropyrons* and winter wheats tend to be prostrate, under cool conditions, while spring wheats tend to be erect; the hybrids approach the *Agropyron* condition. At maturity the *Agropyron* stems are somewhat spreading (mature posture) while *Triticum* stems are erect; with the exception of Mindum × *A. elongatum* which tends to be more erect, the hybrids closely approach the *Agropyron* condition.

The summation of frequencies of the various expressions indicates a dominant or partially dominant influence by the *Agropyron* parent. Hybrids involving *A. elongatum* show a higher degree of *Agropyron* dominance than do hybrids involving *A. glaucum*. This is probably to be expected on the basis of chromosome number of the *A. elongatum* pollen ($n = 35$) as compared to *A. glaucum* pollen ($n = 21$), especially since considerable autosyndesis occurs among *A. elongatum* chromosomes (8). Of the *Triticum* parents, the variety C.A.N. 1835 appears to have been least able to overcome *Agropyron* dominance.

Considering the characters individually, the expressions are for the most part either dominant or partially dominant *Agropyron*. There are, however,



1. *Unstimulated and stimulated ovaries of Crail Fife pollinated with A. junceum.* 2. *Hybrid seeds from Mindum pollinated with A. elongatum.* 3. *Root systems extracted from special containers. (Note yard stick). Left to right, A. elongatum (second year), Kharkov × A. elongatum (second year), Kharkov (fall sown, mature).*

TABLE VII

DESCRIPTIONS OF CERTAIN F_1 PLANTS SUMMARIZED IN TERMS OF RELATIVE DEGREES OF DOMINANCE, TRANSGRESSIVE EXPRESSION, OR HYBRID VIGOR

Character	*A. glaucum*					*A. elongatum*					Total
	Lutescens 0.62	C.A.N. 1835	Kharkov	Mindum	Vernal emmer	Lutescens 0.62	C.A.N. 1835	Kharkov	Mindum	Vernal emmer	
Life tenure	A	A	A	A	A	A	A	A	A	A	
Vegetative period	XA, H	XA	XA, H	XA	XA, H	XA, H	XA, H	XA, H	XA, H	XA, H	
Time of flowering	IA	IA	A	IA	IA	IA	IA	A	IA	IA	
Heading-to-flowering period	A	A	A	A	A	A	A	A	A	A	
Posture juvenile	IA	IA	I	IA	IA	IA	IA	I	IA	IA	
Posture mature	IA	A	A	A	IA	A	A	A	I	IA	
Height	H	–	I	H	H	H	H	H	–	H	
Tillering	H	A	A	X	H	H	A	H	A	H	
Leafiness	H	IA	IA	X	H	IA	IA	IA	H	IA	
Hollowness or solidity of stem	A	IA	IT	I	IA	IA	IA	IA	IA	I	
Reaction to ergot	XA	A	XA	A	XA	–	–	–	–	–	
Seminal rootlet number	IA	IA	IA	IA	IA	IA	IA	IA	IA	IA	
Mature root, extent	–	–	–	–	A	A	–	A	–	A	
Mature root, texture	–	–	–	–	I	I	–	I	–	I	
Coleoptile color	–	IA	IA	IA	IA	–	IA	IA	IA	IA	
Shattering of rachis	IA	IA	IA	IA	–	IA	IA	IA	IA	–	
Adherence of glumes to seed	–	–	–	–	–	IA	IA	IA	IA	IA	
Spike density	IT	I	I	I	I	I	IA	IA	IA	IA	
No. of spikelets	A	A	A	A	I	A	A	A	XA	XA	
No. of florets	A	A	A	A	A	IA	IA	IA	T	IA	
Awning	I	A	IA	IA	IA	A	A	IA	IA	A	
Secondary glume shoulder	A	A	IA	–	–	IA	–	I	–	XA	
Secondary glume beak length	T	A	I	A	T	I	A	I	I	I	
Secondary glume keel	T	X	T	T	T	A	A	I	A	I	
Leaf length	I	XA	–	–	A	IA	IA	–	–	A	
Leaf width	IA	IA	I	IA	I	IA	IA	IA	A	A	
Leaf pubescence dorsal	IT	IA	IT	IT	I	I	I	–	–	XT	
Leaf pubescence ventral	I	XT	IT	IT	I	I	I	IA	I	I	
Leaf pubescence marginal	IA	I	I	I	IA	I	I	X	I	I	

Concluded on page 434

TABLE VII—*Concluded*

DESCRIPTIONS OF CERTAIN F_1 PLANTS SUMMARIZED IN TERMS OF RELATIVE DEGREES OF DOMINANCE, TRANSGRESSIVE EXPRESSION, OR HYBRID VIGOR—*Concluded*

Character	*A. glaucum*					*A. elongatum*					Total
	Lutescens 0.62	C.A.N. 1835	Kharkov	Mindum	Vernal emmer	Lutescens 0.62	C.A.N. 1835	Kharkov	Mindum	Vernal emmer	
Leaf venation number	IA	IA	I	IA	IA	I	IT	I	I	I	
Leaf venation barbing dorsal	I	IA	–	I	IT	A	X	–	–	A	
Leaf venation barbing ventral	–	–	–	–	T	–	–	A	–	I	
Leaf marginal barbing	A	XT	IT	I	IA	A	A	XA	IA	IA	
Leaf rigidity	I	IT	–	I	I	I	I	I	IA	I	
Auricle color	–	–	T	–	–	–	–	T	–	–	
Ligule length	IA	IA	A	I	I	A	A	IA	A	A	
Node number	H	–	I	I	I	A	A	A	IA	T	
Node pubescence	A	A	IA	–	A	A	A	IA	–	A	
Culm diameter	I	IA	I	IA	I	A	A	A	XA	XA	
Neck diameter	I	IA	I	–	I	A	A	A	X	X	
Frequencies of the various expressions:											
Dominance: *Agropyron* (A)	8	11	8	7	6	14	14	10	6	9	93
Intermediate—*Agropyron* (IA)	9	14	7	9	10	11	12	13	12	10	107
Intermediate (I)	7	2	10	8	11	8	4	7	5	9	67
Intermediate—*Triticum* (IT)	2	1	4	2	1	0	1	0	0	0	15
Triticum (T)	2	0	2	1	3	0	0	1	1	1	11
Total under dominance	28	28	31	27	31	33	31	31	24	29	293
Transgressive: indeterminate (X)	0	1	0	2	0	0	1	1	1	1	7
Agropyron (XA)	2	2	2	1	2	1	1	2	3	4	20
Triticum (XT)	0	2	0	0	0	0	0	0	0	1	3
Hybrid vigor (H)	5	0	1	1	4	3	2	3	2	3	24

a number of cases where the expression is for the most part intermediate, for example, spike density, leaf marginal pubescence, leaf rigidity, node number, culm and neck diameter in *A. glaucum* crosses; and beak length, leaf pubescence dorsal, ventral and marginal, venation number and leaf rigidity in *A. elongatum* crosses. There are only two characters for which *Triticum* can be said to express dominance, namely, secondary glume keel in *A. glaucum* crosses and auricle color in crosses of both types. (Auricle color was white in all parental materials except Kharkov, in which the auricle was edged with purple. The purple color was inherited as a dominant).

Transgressive expressions sometimes typify a character, for example, prolongation of vegetative period (probably related to hybrid vigor) in both *A. glaucum* and *A. elongatum* crosses, reaction to ergot in *A. glaucum* crosses, and culm and neck diameters in *A. elongatum* crosses involving tetraploid wheats.

Hybrid vigor is mainly confined to the quantitative characters, height, leafiness and tillering, and to prolongation of the vegetative period (which is considered to be essentially a condition of increased vigor).

Fertility in F_1 Hybrids

During the winter of 1935–1936, 291 F_1 hybrids were grown to maturity in the greenhouse and the spikes examined for seed. Percentage seed-set was expressed on the basis of the number of fully formed florets. The results are summarized in Table VIII.

TABLE VIII

SEED-SET DATA FROM F_1 PLANTS GROWN IN THE GREENHOUSE 1935–1936

Cross	No. of plants	No. of spikes	No. of florets	No. of seeds	Per cent seed-set	Per cent fertile plants	Range of fertility
Kharkov × *A. glaucum*	36	67	1,805	0	0	0	—
Lutescens 0.62 × *A. glaucum*	21	58	1,497	0	0	0	—
C.A.N. 1835 × *A. glaucum*	5	14	368	0	0	0	—
Mindum × *A. glaucum*	53	130	3,299	0	0	0	—
Vernal emmer × *A. glaucum*	53	241	6,537	0	0	0	—
Combined crosses with *A. glaucum*	168	510	13,506	0	0	0	—
Kharkov × *A. elongatum*	38	85	1,762	0	0	0	—
Lutescens 0.62 × *A. elongatum*	16	37	869	0	0	0	—
C.A.N. 1835 × *A. elongatum*	13	33	661	24	3.6	7.7	0 – 27.3
Mindum × *A. elongatum*	5	6	94	0	0	0	—
Vernal emmer × *A. elongatum*	51	30	386	0	0	0	—
Combined crosses with *A. elongatum*	123	191	3,772	24	0.6	0.8	0 – 27.3

The data in Table VIII show that no selfed seeds were obtained from F_1 plants, 168 in number, of crosses involving *A. glaucum*. Another dehiscence was not observed in any of these plants. One seed was obtained from a hybrid of Kharkov × *A. glaucum*; but since no dehiscence occurred on the plant and there was opportunity for accidental backcrossing, it is believed

that the seed originated through backcrossing. The plant grown from the seed in question failed to head the following summer and died during the following winter.

In crosses involving *A. elongatum*, one plant (S4) among C.A.N. 1835 hybrids shed pollen freely and produced 24 seeds, giving a percentage of 27.3. All other plants showed complete non-dehiscence of anthers and no further seed was set.

After the hybrids growing in the greenhouse had been examined for seed they were cut back, cloned, and transplanted to the field in the spring of 1936. Under field conditions the plants grew more vigorously, tillering well and producing large spikes in great abundance. (An exception was the cross Mindum × *A. glaucum*, which grew very poorly in the field.) In crosses involving *A. elongatum* and C.A.N. 1835, Lutescens 0.62, and Kharkov, anther dehiscence in varying degrees was observed to be fairly general. As in the greenhouse, anther dehiscence was not observed among hybrids involving *A. glaucum*. The seed-set data are summarized in Table IX. Percentage seed-set is expressed on the basis of number of spikelets.

TABLE IX

SEED-SET DATA FROM F_1 PLANTS GROWN IN THE FIELD IN 1936

Cross	No. of plants	No. of spikes	No. of spikelets	No. of seeds	Per cent seed-set	Per cent fertile plants	Range of fertility
Kharkov × *A. glaucum*	23	1,768	29,324	0	0	0	—
Lutescens 0.62 × *A. glaucum*	25	2,694	39,334	0	0	0	—
C.A.N. 1835 × *A. glaucum*	4	281	4,342	0	0	0	—
Mindum × *A. glaucum*	3	262	4,192	0	0	0	—
Vernal emmer × *A. glaucum*	88	6,547	101,474	0	0	0	—
Combined crosses with *A. glaucum*	143	11,552	178,666	0	0	0	—
Kharkov × *A. elongatum*	36	3,560	49,973	146	0.3	30.6	0– 20.7
Lutescens 0.62 × *A. elongatum*	24	2,670	36,253	4,260	11.8	75.0	0–118.4*
C.A.N. 1835 × *A. elongatum*	13	3,130	47,054	3,549	7.5	84.6	0– 62.7
Mindum × *A. elongatum*	3	240	4,282	0	0	0	—
Vernal emmer × *A. elongatum*	37	2,225	28,800	0	0	0	—
Combined crosses with *A. elongatum*	113	11,825	166,362	7,955	4.8	35.4	0–118.4

* *The occurrence of percentages of fertility of over 100 is due to the fact that fertility percentages are calculated on the basis of number of spikelets rather than number of florets.*

A very considerable increase in fertility of field material over greenhouse material is demonstrated by the data in Table IX. The crosses, Kharkov × *A. elongatum*, Lutescens 0.62 × *A. elongatum*, and C.A.N. 1835 × *A. elongatum* show marked increases as follows: in seed-set, from 0 to 0.3%, 0 to 11.8%, and 3.6 to 7.5%, respectively; in fertile plants, from 0 to 30.6%, 0. to 75.0%, and 7.7 to 84.6%, respectively; and in upper limit of range of fertility, from 0 to 20.7%, 0 to 118.4%, and 27.3 to 62.7%. (It should be noted that in the case of C.A.N. 1835 × *A. elongatum*, percentage seed-set was based on number of florets in calculating greenhouse data and on number of spikelets in calculating field data.)

The hybrid plant from C.A.N. 1835 × *A. elongatum*, designated as S4, which produced seed in the greenhouse, had been cloned into six individual plants, which under field conditions produced a total of 273 spikes, 4,256 spikelets and 2,670 seeds (seed-set, 62.7%). This was the second-highest percentage seed-set obtained, the highest being from a Lutescens 0.62 × *A. elongatum* hybrid, designated S22, which produced, from a clone of four plants, a total of 129 heads, 1,548 spikelets and 1,833 seeds (seed-set, 118.4%).

The great increase of anther dehiscence observed under field conditions as compared to greenhouse conditions is believed to be due to *(a)* the advantageous effect upon pollen-grain development of the more nearly optimum conditions of nutrition, illumination, etc., obtained in the field and to *(b)* the effect upon anther-wall breakage of such conditions as wind movement, alternations in humidity and temperature, etc., which are more pronounced in the field than in the greenhouse.

In 1937, further data on fertility were obtained in the field upon material identical with, comparable to, or in addition to that previously grown. The fertility data compiled on crosses involving *A. elongatum* are considered to be reasonably comparable to those collected in previous years, since the material was, in many cases, identical and in other cases directly comparable. The results are given in Table X.

TABLE X

SEED-SET DATA FROM F_1 PLANTS GROWN IN THE FIELD IN 1937

Cross	No. of plants	No. of spikes	No. of spikelets	No. of seeds	Per cent seed-set	Per cent fertile plants	Range of fertility
Dawson's G.C. × *A. glaucum*	85	2,742	44,205	0	0	0	—
Minturki × *A. glaucum*	3	35	490	0	0	0	—
Minhardi × *A. glaucum*	13	179	2,957	0	0	0	—
C.D. 1435 × *A. glaucum*	22	434	5,696	0	0	0	—
White Odessa × *A. glaucum*	89	2,387	44,036	0	0	0	—
Kharkov × *A. glaucum*	16	313	5,556	0	0	0	—
Lutescens 0.62 × *A. glaucum*	7	104	1,490	0	0	0	—
Black Persian × *A. glaucum*	12	202	3,588	0	0	0	—
Mindum × *A. glaucum*	32	398	5,454	0	0	0	—
Vernal emmer × *A. glaucum*	45	1,273	17,393	0	0	0	—
T. polonicum × *A. glaucum*	2	76	852	0	0	0	—
Combined crosses with *A. glaucum*	326	8,143	131,717	0	0	0	—
Kharkov × *A. elongatum*	25	5,239	88,636	796	0.9	60.0	0 – 77.8
Lutescens 0.62 × *A. elongatum*	21	996	12,217	2,702	22.1	57.1	0 – 134.7
C.A.N. 1835 × *A. elongatum*	8	201	2,424	993	41.0	37.5	0 – 124.8
Mindum × *A. elongatum*	5	311	5,200	210	4.0	80.0	0 – 49.6
Vernal emmer × *A. elongatum*	109	7,370	94,125	400	0.4	33.0	0 – 11.2
Combined crosses with *A. elongatum*	168	14,117	202,602	5,101	2.5	41.7	0 – 134.7

The most striking feature of the 1937 results is the seed-sets obtained from the *A. elongatum* crosses involving the tetraploid wheats, Mindum and Vernal emmer, which previously had been completely sterile. Most of this material had grown to maturity the previous years without setting seed. A possible

explanation is that the plants did not become properly established early enough in the first year to permit coincidence between a certain developmental stage of the plant and a critical photoperiod to have a determinative influence on seed-set. Accidental backcrossing is not believed to be a tenable explanation.

In the *A. elongatum* crosses involving Lutescens 0.62 and C.A.N. 1835, made up of new clonal material from plants used in previous years, there was a marked increase in percentage seed-set and, paradoxically, a very considerable decrease in percentage of fertile plants. The two hybrid plants, S4 and S22, which were outstanding for fertility in 1936, retained their positions. In 1937, S4 gave 124.8 and S22 gave 134.7% seed-set, both figures representing increases over the previous year.

As previously, there was no definite indication of self seed-set in *A. glaucum* crosses, though a few seeds, thought to be backcrosses, were collected.

Cytological observations reported by Peto (8) show that bivalent and multivalent chromosomal associations are much more numerous in hybrids involving *A. elongatum* than in hybrids in which *A. glaucum* is concerned. This cytological difference is considered to be related to the characteristic fertility and sterility of the two groups of hybrids.

Relation of Pollen Viability to Anther Dehiscence and Fertility

Microscopic observations were made on the pollen of a wide range of F_1 plants in order to determine the relative proportions of functional and non-functional pollen grains produced by each plant. Since seed-set was known to be lower in the greenhouse than in the field for a given plant, pollen from cloned material grown both in the greenhouse and in the field was studied in several instances. These data were then studied in relation to anther-dehiscence and fertility data compiled from the same plants.

Anthers were collected a day or two prior to maturity, killed and fixed in Navashin's agent, washed, run up to 70% ethyl alcohol for storage, and stained as temporary preparations in Belling's iron-aceto-carmine. Five counts were made on five different anthers from each plant.

Data on type of pollen, anther dehiscence, and fertility from five selected plants are summarized in Table XI. The term "good" was applied to pollen grains normal in size and in nuclear and cytoplasmic contents. All other grains were considered as "bad".

It may be concluded from Table XI that: *(a)* there is a far greater proportion of bad pollen in plants grown in the greenhouse than in clones of the same plants grown in the field. *(b)* There is a correlation between dehiscence and the ratio of good to bad pollen. Ratios of 1 : 2.4 and lower proportions of bad give dehiscence, while ratios of 1 : 4.1 and higher proportions of bad give non-dehiscence. *(c)* There is a direct correlation between proportions of good pollen and seed-set when the proportions are sufficient to give anther dehiscence.

TABLE XI

DATA ON THE PROPORTIONS OF GOOD AND BAD POLLEN IN COMPARISON WITH ANTHER DEHISCENCE AND PERCENTAGE SEED-SET

Material (F_1)	Where grown	Type of pollen	Pollen counts					Total	Ratio good : bad	Condition of anther	Per cent seed-set
			1	2	3	4	5				
C.A.N. 1835 × *A. elongatum* (S4)	Greenhouse	Good	154	130	137	265	330	1016			
		Bad	500	500	500	465	500	2465	1 : 2.4	Dehiscent	27.3
C.A.N. 1835 × *A. elongatum* (S4)	Field	Good	575	770	518	647	638	3148			
		Bad	150	200	150	200	200	900	1 : 0.3	Dehiscent	108.9
Lutescens 0.62 × *A. elongatum* (S22)	Greenhouse	Good	0	0	0	0	0	0			
		Bad	500	500	500	500	500	2500	1 : ∞	Non-dehiscent	0
Lutescens 0.62 × *A. elongatum* (S22)	Field	Good	637	511	122	592	430	2292			
		Bad	398	252	98	341	500	1589	1 : 0.7	Dehiscent	118.4
Lutescens 0.62 × *A. elongatum* (S23)	Greenhouse	Good	79	53	243	95	98	568			
		Bad	500	203	500	500	500	2303	1 : 4.1	Non-dehiscent	0
Lutescens 0.62 × *A. elongatum* (S23)	Field	Good	294	787	221	1010	819	3131			
		Bad	160	500	82	500	300	1542	1 : 0.5	Dehiscent	63.6
Dawson's G.C. × *A. glaucum* (32)	Greenhouse	Good	28	14	15	23	17	97			
		Bad	500	276	500	500	500	2276	1 : 23.5	Non-dehiscent	0
Dawson's G.C. × *A. glaucum* (32)	Field	Good	34	33	49	37	38	191			
		Bad	500	430	500	500	425	2355	1 : 12.3	Non-dehiscent	0
Lutescens 0.62 × *A. elongatum* (S17)	Greenhouse	Good	388	118	194	9	144	823			
		Bad	412	500	424	500	500	2336	1 : 28.4	Non-dehiscent	0
Lutescens 0.62 × *A. elongatum* (S17)	Field	Good	435	416	0	510	292	1653			
		Bad	539	500	500	500	500	2539	1 : 1.5	Dehiscent	2.2

It is believed that internal pressure caused by expansion of the mass of developing pollen grains is an important factor in anther dehiscence. When there is a relatively large proportion of large rigid cells (good pollen) sufficient pressure is exerted to give dehiscence; but when there is a smaller proportion of these cells the pressure is less, and even further diminished by the collapse of the less rigid cells (bad pollen), now in greater proportion. The combined effect is the reduction of internal pressure below the point where it is an effective factor in anther dehiscence.

On the basis of the above data, together with cytological data from Table I of Peto's report (8), tentative statements may be made on the mechanism involved in the fertility-sterility relation. In crosses involving *A. elongatum*, which are more or less fertile, the proportions of bivalent and multivalent chromosomal associations are much higher than in crosses involving *A. glaucum*, which are sterile. The degree of chromosomal association is directly related to the degree of efficiency with which chromosomes are distributed to daughter cells in meiotic divisions, which in turn is directly related to the proportions of good and bad pollen produced, and these in turn are, as indicated above, related to anther dehiscence. In *A. glaucum* crosses, the degree of chromosomal association is below the minimum value required to set into effective operation the series of events leading to anther dehiscence. *A. elongatum* crosses are fertile in proportion to the degree of efficiency given this series of events by the different degrees of chromosomal association.

General Discussion with Particular Reference to Russian Investigations

The primary objective of the *Triticum-Agropyron* hybridization project is the production of new and superior types of forage plants adapted to the drier areas of western Canada. Such plants should be large-seeded to facilitate seeding and harvesting; they should have extensive fibrous root systems with good soil binding properties; they should be perennial and resistant to drought and cold; and they should give a good yield of palatable, nutritious forage. The parents used in these crosses embrace, collectively, all of these qualities. It remains to be seen, however, how near it will be possible to combine ideally these characteristics through selection in the segregating generations. Theoretically, since the perennial habit and clonability of the F_1 plants permit unlimited F_2 populations, only sterility and close genetic linkages stand in the way of the ultimate attainment of this objective.

Besides the primary objective, a number of other breeding possibilities are being kept in mind for future work, mainly with the co-operation of cereal workers. It will suffice here to enumerate these as follows: (i) the production of a perennial wheat that may be bred for feed-grain purposes and perhaps eventually for milling purposes; (ii) the production of a biennial wheat that, either directly or through further crossing, may provide a winter wheat of hardiness superior to existing varieties; and (iii) the isolation of segregate types that may be used as breeding materials for general cereal improvement along the lines of disease resistance, drought resistance, strength of straw, etc.

The original investigation on *Triticum-Agropyron* hybrids began at the Central Station of Plant Breeding and Genetics, Saratov, Russia, in 1930, when N. V. Tzitzin obtained the first hybrids (17). With the creation of perennial wheats as the objective, the work was soon being carried on at several stations in different agricultural regions of the U.S.S.R. To date a score or more of different workers have published results on various phases of the general program of perennial wheat breeding. Without attempting a complete summarization, this older and more extensive work will be considered briefly in comparison with our results and in relation to the prospects of attaining our objectives.

The Russian workers have been successful in crossing five *Agropyron* species with *Triticum*, namely, *A. glaucum*, *A. elongatum* (two forms, with chromosome complements of $2n = 56$ and $2n = 70$, respectively), *A. trichophorum*, *A. junceum* and *A. repens* (11). In early reports *A. intermedium* was also mentioned, but this form was later found to be identical with *A. glaucum* (14). We have been unable to obtain crosses with *A. repens*, although a total of 1,442 *Triticum* florets have been cross-pollinated. We have not tried *A. trichophorum*.

The Russian descriptions of F_1 plants resemble those of the present paper, although greater emphasis is given to *Agropyron* dominance by some authors (13, 17). The great variation among F_1 plants of the same cross is attributed to heterozygosity of the *Agropyron* parents (2, 14), which has led to the use of individual *Agropyron* plants in hybridization (11). The Russian observations on relative fertility among F_1 plants of various crosses agree essentially with the present report (2, 14, 15).

Studies in the U.S.S.R. upon advanced generations indicate that there are excellent possibilities of obtaining constant, perennial forms of superior drought and winter resistance that are earlier than annual wheat and that give high yields of good quality grain. The possibility of obtaining reasonably true-breeding, perennial lines possessing important *Triticum* characteristics was in 1934 demonstrated in an advanced, selfed generation of *T. vulgare* × *A. elongatum* (15). The problem then became one of isolating better-quality types among such lines.

Tzitzin (10) reported the isolation in 1934–1935 of constant, good-yielding, perennial lines with wheat-like seeds. In their second year of growth these lines ripened much earlier than annual wheats, indicating possibilities as a crop for agricultural zones of short growing season. Many of the hybrids inherited the extensive *Agropyron*-type root and proved to be more drought resistant and winter hardy than standard wheats. There were indications that hybrids involving *A. elongatum* had inherited the alkali-tolerance characteristic of this species.

Selecting presumably from backcross generations, Veruschkine (16) obtained several lines of constant, annual wheat types which were cytologically stable ($2n = 42$), markedly disease resistant and characterized by unusually high protein content of the grain (up to 21%). The bread baked from bulked

hybrid grain was considered to be superior to that baked from the standard wheat, Lutescens 0.62, while bread from certain individual hybrids was much higher in quality.

Veruschkine (16) also described intermediate types that, although dying in the field at Saratov, have the capacity to renew growth, indicating that they might behave as perennials in a milder climate. These forms represent a new octoploid wheat ($2n = 56$). They are disease resistant and, though smaller seeded, give higher yields than standard wheats. The protein content ranged from 22.3 to 24.5% in different lines, as compared with 17.2% for Lutescens 0.62, the standard wheat.

In accordance with Meister's belief, Veruschkine (16) considers that the origin of new types among these hybrids is not merely a result of genetic recombination, but also of mutation induced by the remote nature of the crosses.

Biochemical studies by Samsonov (9) and others have shown that the *Agropyron* parents have a much higher gluten content than standard wheats, and that *Agropyron* flour produced a loaf of normal wheat quality. The flour from high-yielding, annual, F_5 hybrids of Lutescens 0.62 × *A. glaucum* proved to be of excellent baking quality. These tests were considered to indicate that hybrids might be obtained that would replace standard varieties for milling, and it was suggested that biochemical examination of hybrid lines should precede selection work.

Udoljskaja (12) established the existence of two biological types of drought resistance in wheat. In plants of Type I, under deficient water supply, there is a loss of turgor and retarded development in the form of deferred shoots. Upon resuming adequate watering the deferred shoots revive, the plants recover rapidly, develop at an increased rate and mature with the controls. This type is very resistant at the tillering stage but very susceptible at the heading stage. Plants of Type II, under deficient water supply, lose their leaves, maintain turgor in the main stem, and do not show retarded development. This type is fairly resistant at the tillering stage and very resistant at later stages.

Studying hybrid plants growing in culture and in the field, Udoljskaja found that *Agropyron* dominance in the F_1 hybrids masked the type of resistance inherited from wheat. These types of resistance were expressed in later generations, however, and among the F_6 hybrids a number of lines were found to be superior to wheat in drought resistance. Hybrids involving winter wheats possessed Type I resistance.

Blinkova (3) studied winter hardiness in parental *Agropyron* species and concluded that they possessed exceptional winter hardiness owing to several physiological and biochemical properties that are transmitted in varying degrees to the *Triticum-Agropyron* hybrids. Analysis of the hybrids shows that *(a)* they are characterized by resistance of plasmic proteins to low temperature and by mobility of the albumen complex; *(b)* they are more

tolerant of conditions of hardening than the most hardy wheat and rye; and *(c)* they expend reserve nutrient with greater economy and secrete carbohydrate more vigorously during hardening than do hardy wheat and rye. Hybrids that failed to head during the first year after sowing were found to have high plasmic resistance to low temperature. It was concluded that there is a correlation between developmental stages and changes in plasmic proteins.

Although the Russian investigations have not been conducted from the forage point of view, it is inevitable that the results should have important bearings upon a forage-breeding project based on the same crosses. The frequent isolation by Russian workers of constant, fertile, resistant and highly-productive perennial forms is extremely important to the forage project because they are qualities toward which forage selection must be directed. The Russian work on drought and winter resistance applies directly to forage breeding. The high protein content of the grain of Russian selections indicates possibilities of developing a forage plant which might yield also a valuable grain-concentrate for feeding purposes. There remains unanswered, however, the very important question of the possibility of isolating suitable forage types. It can only be said that there is nothing in the Russian literature nor in the present report that should discourage a full attack upon the forage-breeding problem.

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THE EFFECT OF DILUTION AND DENSITY ON THE FERTILIZING CAPACITY OF FOWL SPERM SUSPENSIONS[1]

BY S. S. MUNRO[2]

Abstract

The effect of variations in dilution and numbers of fowl sperm injected into Brown Leghorn hens has been observed in a study of artificial insemination. Fertilization is influenced by the number of sperm introduced in a single injection into the vagina. The number of fertile eggs subsequently produced is affected when the number of sperm is about one hundred million or less, and none are fertile when the number falls below one million.

A synthetic diluent containing sodium sulphate, glucose, and peptone supported motility *in vitro*, but affected the fertilizing capacity of fowl sperm in proportion to the percentage occurrence of the diluent in the suspension. Sperm serum, comprising about 75% of normal fowl semen, produces little harmful effect and appears to be a more favorable diluent. However, the development of the zygotes conceived by the sperm surviving an unfavorable medium is not impaired, and "hatchability" of the eggs remains unaffected.

By comparison of Walton's data for the rabbit with those reported here for the fowl, it is shown that rabbit sperm are more highly resistant to the "toxicity" of certain types of synthetic fluids. Also, it apparently requires 100 times more sperm in the fowl than in the rabbit for conception to result; this may be related to the comparative anatomy of the reproductive tracts.

Introduction

As a result of investigations in which rabbit sperm secured from the *cauda epididymis* were diluted, in varying degree, with an isotonic sodium chloride solution and tested for fertility by artificial insemination, Walton (11) suggested that fertility rested on a quantitative basis. That is, the chances of conception following coition, as well as the size of the litter produced, depends not only on the number of ova shed but on the number of sperm available for their fertilization. Since the individual sperm become widely dispersed throughout the reproductive tract of the female, the chance of any one being in the vicinity of a fertilizable egg at the crucial time naturally depends on the total number injected. Consequently, there must be a point below which fertility commences to drop. Walton found that fertility was reduced when the number of sperm injected was less than one million, and complete sterility resulted when the number was less than ten thousand. He lists three factors that may have affected the results: "(i) The probability of any one spermatozoon reaching the fertilizable ovum is small; (ii) the spermatozoa are variable

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Contribution from the Poultry Division, Experimental Farm, Ottawa, and the Institute of Animal Genetics, Edinburgh University, Scotland.

[2] *Assistant Agricultural Scientist, Experimental Farms Branch, Ottawa.*

and not all capable of fertilization; (iii) toxicity may act differentially on sperm suspensions of varying density".

The first of these factors is the subject of this investigation. The attempt is made to prove that fertilization is less frequent when the number of sperm injected falls below a certain value, and to determine the approximate level at which no fertilization is obtained.

The second factor should not affect the results, since the proportion of non-functional sperm in the original sample remains the same, regardless of dilution.

As the sperm are suspended in a synthetic diluent that probably is not the most favorable environment, the third factor is most likely to have an effect on the results. If the functional efficiency of the sperm is affected by an unfavorable environment, it is only reasonable to suppose that an increasing proportion of the sperm would be rendered non-functional as the dilutions are increased. This might contribute to the decrease and ultimate cessation of fertilization as much as the decreased numbers of sperm. Since the amount of suspension injected in Walton's experiment was always the same, he was not able to measure separately the effect of dilution and of sperm number.

This question is of great importance, not only from the standpoint of practical artificial insemination, but also because of its bearing on the scientific concept of fertilization. Since Walton's results cannot be applied to the fowl, an experiment was designed to establish the degree to which fowl sperm can be diluted before fertility is affected. This must be known, approximately, before artificial insemination can be used intelligently as a tool for investigating the fundamental aspects of fertilization.

The fowl offers many advantages over the laboratory rodent as experimental material in the investigation of this problem. The hen remains consistently in oestrus or "in the lay" for long periods, and as fertilization normally follows mating at any time of day, one is not confronted with the necessity of synchronizing insemination and ovulation. This is rather difficult to accomplish in the rodent; and even in the rabbit, where ovulation can be induced by copulation with a vasectomized buck, it is often difficult to secure a number of does that will accept the buck at the same time. Furthermore, one copulation in the fowl serves to fertilize eggs for an average of about two weeks, and one can thus measure not only the production of fertile eggs within a given time limit, but also the length of time fertilization continues. In addition, the technique of artificial insemination in the fowl has been rendered very simple and efficient through the discovery by Burrows and Quinn (1) of the abdominal stimulation method of eliciting an ejaculatory reflex in the cock, and by the author's method (Munro (7)) of everting the vagina of the female to introduce sperm directly into the uterus. Finally, the male fowl possesses no accessory sex glands; the sperm are thus collected free from secondary secretions and have no tendency to coagulate as do the natural ejacula of rodents.

Materials and Methods

The experiment, as originally planned, utilized six males and a group of about 60 females. The males were fully matured, about nine months old, at the beginning of the experiment. They had been regularly submitted to induced ejaculations during the preceding six weeks. The females were slightly younger and were just reaching the peak of their egg production. All birds were pure-bred Brown Leghorns, bred and reared at the Institute of Animal Genetics, Edinburgh.

All glassware used was thoroughly cleaned and neutralized. Owing to the small volume of the ejacula emitted by the cock (averaging about 0.5 cc. in the stock used) a rather delicate technique is required to effect the dilutions. After a few preliminary trials, it was found that they were most easily and accurately made by the drop method. A series of eight test tubes was used, and the semen was transferred from the small collecting dish to the first test tube by means of a finely graduated 1-ml. pipette with an attached rubber teat, the volume being measured and the number of drops counted in the process. The pipette was then thoroughly flushed with distilled water and with the diluent. Into each of the remaining seven test tubes were then delivered from two to four drops of the diluent; the actual number of drops depended on the volume of semen collected, but was the same in all tubes. The semen in the first tube was then thoroughly mixed by shaking, and a number of drops equal to that of the diluent was added with the same pipette to the first of the diluent-containing tubes. The thin layer of pure semen on the inner surface of the pipette was removed by flushing with distilled water followed by the diluent, and the contents of the test tube was then thoroughly mixed by sucking in and out of the pipette several times. This provided a 1 : 1 mixture of semen and diluent, or a 1/2 dilution. Likewise, by transferring from this tube an equal volume of the 1/2 dilution to the following tube, a 1/4 dilution was obtained. In this way an ascending series of dilutions—undiluted, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and 1/128—each containing half as many sperm per unit volume as the preceding, was obtained.

In a series of preliminary trials it was shown, by counting with a Thoma-Hawksley haemocytometer, that the actual sperm density of each of these dilutions could be accurately predicted by estimations based on the observed density of the 1/2 dilution (Table I). Such estimations were found to be more accurate than those based on the sperm density of pure semen. In each trial, application of the χ^2 test for goodness of fit reveals that the differences between the observed and expected numbers fall within the range of chance. In the actual experiment, therefore, the sperm density of the 1/2 dilution only has been counted, the others being computed from this value.

The diluent used was that recommended by Milovanov (6) for rabbit sperm; it is relatively simple to prepare, supports motility *in vitro* longer than normal saline or Ringer's solution, and as long as the more complicated sodium and potassium phosphate solutions. Equal quantities of the eight dilutions were injected into hens that had laid on the same day, usually within four

or five hours. No eggs, therefore, were present in the uterus to interfere with insertion of the syringe and deposition of the semen. One bird was used for each dilution. The injection was made into the vagina by means of a finely graduated, all-glass, tuberculin syringe without a needle. The glassware was kept slightly warmed on a fibre-board square over an electric heater.

TABLE I

ACCURACY OF ESTIMATING SPERM DENSITY IN A SERIES OF DILUTIONS BY CALCULATION FROM THE OBSERVED DENSITY OF THE 1/2 DILUTION

(Results of applying the χ^2 test are shown)

Trial No.		Pure	Dilution								χ^2	P
			1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256		
I	Observed	384	179	84	37	18	10	5	3	2	2.992	0.89
	Expected	358		89.5	44.7	22.4	11.2	5.6	2.8	1.4		
II	Observed	363	195	126	43	22	11	6	3	–	11.223	0.08
	Expected	390		97.5	48.7	24.4	12.2	6.1	3.0	–		
III	Observed	349	174	85	41	23	13	–	–	–	0.664	0.96
	Expected	348		87	43.5	21.8	10.9	–	–	–		
IV	Observed	328	181	83	40	26	12	–	–	–	4.968	0.29
	Expected	362		90.5	45.2	22.6	11.3	–	–	–		

Following insemination, the hens were trapnested and the eggs were marked and dated. These were placed in an incubator at weekly intervals. On the third day they were examined for fertility, and on the seventh and fourteenth days for dead embryos. By completing the records at hatching time, it was possible to determine if embryos hatched, or died during the first, second, or third weeks of incubation. Thus a record was kept for each egg laid by each hen after the artificial insemination. The protocol of a representative experiment is shown in Table II.

When a hen had produced consecutively five or more infertile eggs she was considered infertile until again inseminated, and was free to be used in a new experiment. A hen very rarely produces a fertile egg when the preceding five have been infertile. Therefore, this source of error can be safely ignored in the present experiment, especially since most of the hens were not used immediately after becoming infertile as indicated by this test.

Records of "hatchability" as well as fertility of the eggs were kept, to indicate the effect of pre-treatment of the sperm on vigor of the resulting embryo, apart from the ability to initiate conception.

All inseminations were performed between three and six p.m. Under these conditions only one egg was fertile out of 81 laid by fertile hens on the

TABLE II

FERTILITY AND "HATCHABILITY" OF EGGS LAID AFTER ARTIFICIAL INSEMINATION

A typical protocol. The fertility and hatchability records are calculated on the data of the 2nd to 11th days, inclusive. I = infertile; H = hatched; D_1, D_2, D_3 = embryo died in 1st, 2nd, or 3rd week of incubation. Amount of fluid injected, 0.05 cc.

Dilution	Millions of sperm per cu. mm.	Hen No.	Days after insemination																		
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Pure	9.54	960	I	I		H	H	H	H		I	H	I	I	I		I		I		I	
1/2	4.77	700	I		H	D_2	D_1		I	H	D_3	H		I	I	I				I		I
1/4	2.38	671	I	I		H	H	H		H	D_2	H			I		I	I		I	I	
1/8	1.19	776	I		H	H	H		H	I		H	H		I	I	I		I	I	I	
1/16	0.60	947	I	I		H	I	H	I		I	D_2	I	I		I	I	I	I		I	I
1/32	0.30	686	I	I	I		I	I		H		I			I	I		I		I		
1/64	0.15	1001	I		I			I		I			I		I	I						
1/128	0.07	731	I	I		I	I			I			I		I			I				

first day following insemination. On the second day 88% of the eggs laid were fertile. This percentage did not increase on the third day. For purposes of comparison, fertility was calculated as the percentage of fertile eggs produced in a ten-day period beginning on the second day following insemination. "Hatchability" is the percentage of fertile eggs that hatched.

Effects on Fertility

(a) Dilution with a Synthetic Diluent

Three experiments were conducted with each of six males. In the first, 0.05 cc. of the various suspensions was injected into each hen, in the second 0.2 cc., and in the third 0.3 cc. By thus varying the total amount of fluid injected and therefore the numbers of sperm, an additional means was provided for determining whether the differences were due to the degree of dilution or to the number of sperm injected. The results of the 18 experiments are shown in Table III. The results have been grouped into three divisions according to the volume injected, and within each division the results are listed by males, the latter being arranged in order of sperm density.

Occasionally a hen ceased to lay after insemination. Such instances account for the few cases where no data are given for a particular dilution. Pauses in egg production in individual birds are frequent, but these pauses are not more common following handling and insemination than at other times. Infertile hens that had laid just previous to insemination were scarce during the latter part of the experiment. When only seven of the desired eight were available, the test of the pure semen was omitted. This accounts for lack of data with the pure semen for five of the six males in the last division of Table III. However, this is immaterial since it is the degrees of fertility at the various dilutions in the three injection levels that are the important comparisons. The table shows that fertility drops rapidly when the semen is diluted. This begins even at the 1/2 dilution, which gives considerably poorer fertility than does pure semen; the decline is steady and reaches the zero point at a dilution of 1/64 in all levels excepting 0.30 cc., where one fertile egg is recorded out of 35 set at 1/128. Since this is the only fertile egg in all the data below 1/32, it can safely be regarded as a purely chance occurrence.

Two things in Table III very definitely indicate that it is not the decrease in total numbers of injected sperm that is responsible for the decrease in fertility with increasing dilution: (i) there is no increase in fertility as the amount of injected fluid increases, and (ii) within each of the three injection levels there is no tendency for fertility to be higher in the suspensions which are naturally denser. The sperm densities of the undiluted semen as estimated from the haemocytometer counts of the 1/2 dilution are shown in the table; since within each division the males are arranged in order of this estimated density, the fertility levels in the divisions should increase as the table descends. This does not occur even when there are five times more sperm in the semen of the last male than in that of the first, as in the second division. As there is little consistency in the density of the sperm samples from the individual

TABLE III

EFFECT OF DILUTION AND SPERM DENSITY OF FOWL SEMEN ON FERTILITY AND HATCHABILITY OF EGGS LAID AFTER ARTIFICIAL INSEMINATION

The numbers in each cell of the table indicate: top row—eggs set : fertile : hatched; bottom row—eggs fertile, % : eggs hatched, %.

		Each hen injected with 0.05 cc. fluid							
Male No.	Millions of sperm per cu. mm.	Pure	1/2	1/4	1/8	1/16	1/32	1/64	1/128
52	2.84	7 : 7 : 7 100 : 100	6 : 4 : 2 67 : 50	5 : 4 : 4 80 : 100	8 : 3 : 3 38 : 100	5 : 0 : 0 0 : –	4 : 0 : 0 0 : –	— —	— —
51	5.48	6 : 4 : 3 67 : 75	6 : 3 : 2 50 : 67	6 : 0 : 0 0 : –	5 : 2 : 2 40 : 100	6 : 0 : 0 0 : –	8 : 0 : 0 0 : –	6 : 0 : 0 0 : –	6 : 0 : 0 0 : –
99	5.78	6 : 5 : 5 83 : 100	7 : 4 : 2 57 : 50	6 : 0 : 0 0 : –	6 : 1 : 1 17 : 100	5 : 0 : 0 0 : –	5 : 0 : 0 0 : –	1 : 0 : 0 0 : –	5 : 0 : 0 0 : –
95	6.46	6 : 6 : 6 100 : 100	6 : 6 : 6 100 : 100	7 : 2 : 2 29 : 100	3 : 0 : 0 0 : –	6 : 0 : 0 0 : –	6 : 0 : 0 0 : –	3 : 0 : 0 0 : –	4 : 0 : 0 0 : –
97	6.85	5 : 5 : 5 100 : 100	7 : 7 : 5 100 : 71	4 : 0 : 0 0 : –	6 : 0 : 0 0 : –	5 : 0 : 0 0 : –	3 : 0 : 0 0 : –	— —	— —
50	9.54	8 : 5 : 5 63 : 100	8 : 6 : 3 75 : 50	6 : 6 : 5 100 : 83	7 : 6 : 6 86 : 100	8 : 3 : 2 38 : 67	5 : 1 : 1 20 : 100	4 : 0 : 0 0 : –	4 : 0 : 0 0 : –
Unweighted mean		85.5 : 95.8	74.8 : 64.7	34.8 : 94.3	30.2 : 100	6.3 : 66.7	3.3 : 100	0 : –	0 : –
Weighted mean		84.2 : 96.9	75.0 : 66.7	35.3 : 91.7	34.3 : 100	8.6 : 66.7	3.5 : 100	0 : –	0 : –

TABLE III—*Continued*

EFFECT OF DILUTION AND SPERM DENSITY OF FOWL SEMEN ON FERTILITY AND HATCHABILITY OF EGGS LAID AFTER ARTIFICIAL INSEMINATION—*Cont.*

The numbers in each cell of the table indicate: top row—eggs set : fertile : hatched; bottom row—eggs fertile, % : eggs hatched, %.

Each hen injected with 0.20 cc. fluid

Male No.	Millions of sperm per cu. mm.	Pure	1/2	1/4	1/8	1/16	1/32	1/64	1/128
50	1.94	7 : 7 : 7	7 : 3 : 1	7 : 2 : 2	6 : 2 : 2	4 : 1 : 1	—	2 : 0 : 0	6 : 0 : 0
		100 : 100	43 : 33	29 : 100	33 : 100	25 : 100	—	0 : –	0 : –
52	3.48	8 : 8 : 8	7 : 4 : 3	5 : 0 : 0	7 : 1 : 1	6 : 0 : 0	6 : 0 : 0	6 : 0 : 0	3 : 0 : 0
		100 : 100	57 : 75	0 : –	14 : 100	0 : –	0 : –	0 : –	0 : –
95	3.94	—	9 : 8 : 8	7 : 4 : 4	7 : 3 : 3	6 : 0 : 0	8 : 1 : 1	2 : 0 : 0	5 : 0 : 0
		—	89 : 100	57 : 100	43 : 100	0 : –	13 : 100	0 : –	0 : –
97	8.37	6 : 6 : 5	—	5 : 3 : 3	4 : 0 : 0	5 : 1 : 1	6 : 2 : 2	4 : 0 : 0	6 : 0 : 0
		100 : 83	—	60 : 100	0 : –	20 : 100	33 : 100	0 : –	0 : –
51	9.01	4 : 1 : 1	5 : 3 : 2	6 : 2 : 1	5 : 0 : 0	7 : 2 : 1	4 : 0 : 0	3 : 0 : 0	5 : 0 : 0
		25 : 100	60 : 67	33 : 50	0 : –	29 : 50	0 : –	0 : –	0 : –
99	9.84	8 : 6 : 6	8 : 0 : 0	6 : 4 : 4	7 : 1 : 0	7 : 0 : 0	7 : 0 : 0	7 : 0 : 0	8 : 0 : 0
		75 : 100	0 : –	67 : 100	14 : 0	0 : –	0 : –	0 : –	0 : –
Unweighted mean		80.0 : 96.6	49.8 : 68.75	41.0 : 90.0	17.3 : 75.0	12.3 : 83.3	9.2 : 100	0 : –	0 : –
Weighted mean		84.8 : 96.4	50.0 : 77.8	41.7 : 93.3	19.4 : 85.7	11.42 : 75.0	9.7 : 100	0 : –	0 : –

TABLE III—*Concluded*

EFFECT OF DILUTION AND SPERM DENSITY OF FOWL SEMEN ON FERTILITY AND HATCHABILITY OF EGGS LAID AFTER ARTIFICIAL INSEMINATION—*Conc.*

The numbers in each cell of the table indicate: top row—eggs set : fertile : hatched; bottom row—eggs fertile, % : eggs hatched, %.

		Each hen injected with 0.30 cc. fluid							
Male No.	Millions of sperm per cu. mm.	Pure	1/2	1/4	1/8	1/16	1/32	1/64	1/128
52	497	5 : 5 : 4 100 : 80	8 : 7 : 5 88 : 71	5 : 0 : 0 0 : –	4 : 0 : 0 0 : –	6 : 0 : 0 0 : –	3 : 0 : 0 0 : –	5 : 0 : 0 0 : –	5 : 0 : 0 0 : –
97	545	— —	6 : 5 : 4 83 : 80	6 : 1 : 1 17 : 100	5 : 0 : 0 0 : –	6 : 0 : 0 0 : –	6 : 0 : 0 0 : –	8 : 0 : 0 0 : –	6 : 0 : 0 0 : –
95	673	— —	7 : 7 : 6 100 : 86	7 : 4 : 4 57 : 100	8 : 2 : 2 25 : 100	6 : 0 : 0 0 : –	8 : 2 : 2 25 : 100	6 : 0 : 0 0 : –	5 : 0 : 0 0 : –
51	684	— —	7 : 6 : 3 86 : 50	8 : 0 : 0 0 : –	6 : 0 : 0 0 : –	6 : 0 : 0 0 : –	7 : 0 : 0 0 : –	6 : 0 : 0 0 : –	6 : 1 : 1 17 : 100
99	820	— —	— —	7 : 0 : 0 0 : –	7 : 2 : 2 29 : 100	8 : 0 : 0 0 : –	6 : 0 : 0 0 : –	5 : 0 : 0 0 : –	5 : 0 : 0 0 : –
50	922	— —	7 : 2 : 0 29 : 0	8 : 0 : 0 0 : –	8 : 2 : 0 25 : 0	8 : 0 : 0 0 : –	5 : 0 : 0 0 : –	7 : 0 : 0 0 : –	8 : 0 : 0 0 : –
Unweighted mean			77.2 : 57.4	12.3 : 100	13.2 : 66.7	0 : –	4.2 : 100	0 : –	2.8 : 100
Weighted mean			77.1 : 66.7	12.2 : 100	15.8 : 66.7	0 : –	5.7 : 100	0 : –	2.9 : 100
General weighted mean		85.5 : 95.4	67.6 : 69.3	28.8 : 93.8	22.9 : 88.0	6.4 : 71.4	6.2 : 100	0 : –	1.1 : 100

males, it might be thought that the technique of counting was at fault. However, the close agreement shown between the expected and the observed counts (Table I) refutes this suggestion.

A detailed discussion of the errors involved in estimating sperm density with the haemocytometer is given in a paper now in preparation. This subject, therefore, will not be discussed here. It may be mentioned that variations in the sperm density of semen samples from the same male occur naturally, and represent true differences rather than technical errors in counting. In fact, the sperm densities listed in the various tables of this paper are essentially correct within the limits of a small technical error and may be regarded as the actual densities. It would appear, then, from the data listed in Table III, that the diluent exerts a harmful effect that is proportional to the degree of dilution regardless of the density of the suspension.

This was critically tested by making three dilutions, 1/4, 1/16, 1/64, by diluting one drop of pure semen with the appropriate number of drops of diluent and injecting the total fluid. It was not possible to insure that the hen would retain the whole volume of higher dilutions after injection. As there was slightly over 3 cc. in the 1/64 dilution, the hen may have ejected a small amount of the fluid after her release, although all was retained at the time. Sperm samples from five different males were diluted in this manner and injected. Of each semen sample, one drop undiluted was also injected, making four levels for comparison. The results are given in Table IV. It is seen that

TABLE IV

EFFECT OF INJECTING EQUAL NUMBERS OF SPERM WITH INCREASING AMOUNTS OF MILOVANOV'S DILUENT

(Data presented as in Table III)

Male No.	Millions of sperm per cu. mm.	Pure	1/4	1/16	1/64
95	6.16	8 : 4 : 2 50 : 50	7 : 1 : 1 14 : 100	7 : 0 : 0 0 : –	6 : 0 : 0 0 : –
50	7.18	6 : 3 : 1 50 : 33	6 : 6 : 6 100 : 100	5 : 1 : 1 20 : 100	7 : 0 : 0 0 : –
52	7.80	6 : 6 : 4 100 : 67	5 : 1 : 0 20 : 0	8 : 1 : 0 13 : 0	4 : 0 : 0 0 : –
51	8.66	8 : 1 : 1 13 : 100	7 : 1 : 1 14 : 100	2 : 0 : 0 0 : –	8 : 1 : 1 13 : 100
97	10.20	8 : 8 : 6 100 : 75	8 : 1 : 0 13 : 0	8 : 0 : 0 0 : –	8 : 1 : 1 13 : 100
Unweighted mean		62.6 : 65.0	32.2 : 60.0	6.6 : 50.0	5.2 : 100
Weighted mean		61.1 : 63.6	30.3 : 80.0	6.7 : 50.0	6.1 : 100

fertility falls rapidly with dilution of the sperm, and although the pure sperm gave lower fertility than is shown in Table III, the other levels are quite comparable. Obviously, the lowering of fertility as a result of dilution is due to differential toxicity, and the results of the first experiment afford no measure of the effect of the decreasing numbers of sperm injected.

Before the latter effect could be tested properly, a more satisfactory diluent was needed. Several other types of synthetic diluents were available for testing, including that especially recommended by Nikitina (8) for fowl sperm. However, as motility is generally recognized as a very good indication of fertilizing ability, and as the diluent used was found to maintain motility as long as any of several widely different types, it seemed unlikely that an ideal synthetic diluent could be obtained. Moreover, the task of testing biologically the efficiency of various diluents by their effects on fertility is rather a prolonged procedure. Therefore, an attempt was made to secure some natural fluid that would be more favorable and that might be used in an investigation of the immediate problem, *viz.*, the quantitative nature of fertilization. For this purpose sperm serum promised to be ideal.

(b) Dilution with Sperm Serum

The pooled semen from 14 or 15 males, comprising about 8 cc., yielded about 6 cc. of sperm-free serum after centrifuging for 15 to 20 min. at 2000 r.p.m. This serum is slightly milky although quite watery in consistency. A very occasional sperm cell may be found in the serum, but any such sperm are included in the estimated density of the dilutions made with serum, and

TABLE V

EFFECT OF INJECTING EQUAL NUMBERS OF SPERM WITH INCREASING AMOUNTS OF SPERM SERUM AS DILUENT

(Data presented as in Table III)

Male No.	Millions of sperm per cu. mm.	Pure	1/4	1/16	1/64
51	4.38	6 : 5 : 3 83 : 60	6 : 6 : 6 100 : 100	5 : 2 : 2 40 : 100	
52	6.02	27 : 19 : 17 70 : 89	28 : 16 : 16 57 : 100	27 : 13 : 12 48 : 92	
97	7.72	11 : 9 : 7 82 : 78	— —	4 : 4 : 2 100 : 50	8 : 4 : 3 50 : 75
95	7.82	14 : 9 : 8 64 : 89	15 : 6 : 5 40 : 83	13 : 7 : 6 54 : 86	
Unweighted mean		74.8 : 79.0	65.7 : 94.3	60.5 : 82.0	50 : 75
Weighted mean		72.4 : 83.3	57.2 : 96.4	53.1 : 84.6	50 : 75

thus do not introduce an error. In order to test the efficiency of the sperm serum a test was run, similar to that described above with synthetic diluent, *i.e.*, a series of three dilutions, 1/4, 1/16 and 1/64, containing the same number of sperm. Samples were secured from four of the males used in the previous test, diluted, and injected. When the volume of semen secured was sufficient, two preparations of each dilution were made and twice the number of hens injected. The results are given in Table V. It can be seen that there is but a slight drop in fertility as the dilutions increase. The results are compared graphically with the synthetic diluent in Fig. 1.

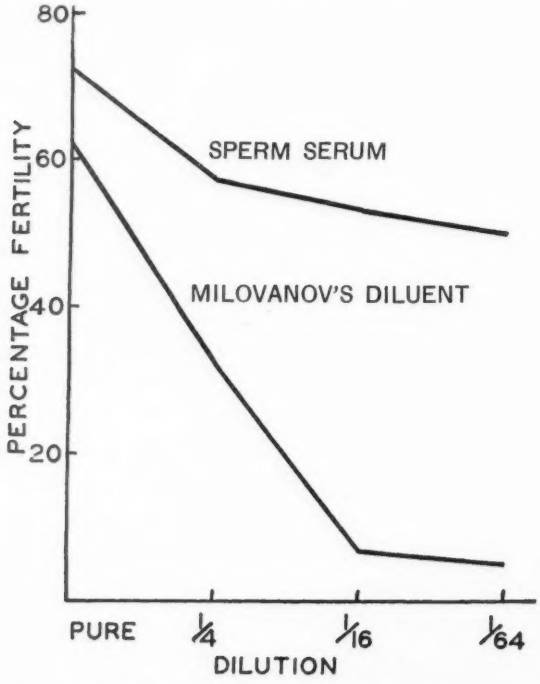

FIG. 1. *Graph showing the superiority of sperm serum to a synthetic fluid when used as a diluent of semen, previous to artificial insemination. Same number of sperm injected at all dilutions.*

Theoretically, equal levels of fertility are expected with the various dilutions. Several explanations may be given for the slight drop manifested. First, sera from different males probably differ slightly in chemical composition and may produce slight adverse effects on foreign sperm cells. Second, small amounts of the larger volumes may have been expelled by the hen. Third, experimental error may be responsible (*e.g.*, only one male was used in the 1/64 dilution). However, the fertility remained relatively constant when

compared to that secured under similar circumstances with the synthetic diluent. It appeared that the effect of decreasing sperm numbers could be measured by using sperm serum as a diluent, and an experiment to test this point was carried out.

By the time the investigation had progressed to this stage, the males had been maintained for nine months in individual cages. Although fed a complete ration they gradually lost appetite and weight, possibly owing to the development of excessively large combs, which interfered somewhat with eating and drinking through the grilled wire cage-fronts. The volume of the ejacula did not materially decrease, but motility tests indicated that the sperm were less vigorous than normally. Consequently, only two of the original males that had maintained their weight were used in this experiment. They were supplemented with three younger males that had been penned for some time on the floor and that were producing an average volume of microscopically normal sperm. The experiment was controlled by dividing the semen from each male into two parts; one was diluted to three levels, 1/4, 1/16, and 1/64, with varying volumes of serum but equal numbers of sperm in each; the other was diluted to the same three levels with equal volumes of serum, so that the total number of sperm in each was directly proportional to the dilution. In the latter, dilutions as low as 1/256 were also made and injected, but in the former it was not possible to inject more than the 3 cc. required in the 1/64 dilution. By comparing the results obtained with the two parts of the sample from each male, all variations in the vigor of different males and different ejacula from the same male can be eliminated, and the observed differences in fertilization ascribed to the numbers of sperm injected. Variations in fecundity were equalized by random selection of the hens. The results are given in Table VI and graphically compared in Fig. 2.

By application of the "Student's" t test to the means for each of the three dilutions in Table VI we get a t value of 4.914, the 0.05 point in Fisher's (2) table of t being 4.303. Thus, differences as great or greater than those observed between the two parts of the same sample, or between the different numbers of sperm injected, would be expected to occur by chance less than once in every 20 repetitions of the experiment.

In Table VI, the sperm density of the pure semen is given in millions per cu. mm., and in the next column this has been converted to the actual numbers injected in the pure semen. In the *a* rows, all dilutions contain the same number of sperm as the pure semen. In the *b* rows, the number of sperm injected decreases as the dilution increases; *i.e.*, in the 1/64 dilution the number of sperm is 1/64 of that given in Row *a* for the undiluted sample. These fractional numbers are not given in the table, except as a mean; they can, however, be readily calculated from the information given. In Fig. 2 they are marked at each of the plotted points.

It can be seen that fertility commences to drop at the first dilution (1/4), although as many as 57,900,000 sperm have been injected. It continues to

TABLE VI

COMPARISON OF THE EFFECTS OF INJECTING A CONSTANT OR A DECREASING NUMBER OF SPERM DILUTED WITH SPERM SERUM

(Data presented as in Table III)

(a—equal numbers of sperm; b—decreasing numbers of sperm)

Male No.		Millions of sperm per cu. mm.	Millions of sperm injected	Pure	Dilutions			
					1/4	1/16	1/64	1/256
98	a	3.87	193.5	13 : 4 : 3	7 : 4 : 4	7 : 1 : 1	5 : 1 : 0	—
					57 : 100	14 : 100	20 : 0	—
	b			31 : 75	7 : 1 : 0	8 : 0 : 0	6 : 0 : 0	3 : 0 : 0
					14 : 0	0 : –	0 : –	0 : –
1160	a	4.14	207.0	6 : 5 : 4	7 : 4 : 4	9 : 5 : 4	6 : 2 : 2	—
					57 : 100	56 : 80	33 : 100	—
	b			83 : 80	8 : 5 : 5	6 : 0 : 0	5 : 0 : 0	5 : 0 : 0
					63 : 100	0 : –	0 : –	0 : –
1663	a	4.22	211.0	12 : 9 : 9	5 : 4 : 2	7 : 4 : 4	7 : 5 : 5	—
					80 : 50	57 : 100	71 : 100	—
	b			75 : 100	7 : 1 : 1	7 : 4 : 2	6 : 1 : 1	—
					14 : 100	57 : 50	17 : 100	—
1695	a	4.84	242.0	3 : 1 : 1	7 : 2 : 2	6 : 1 : 1	5 : 1 : 1	—
					29 : 100	17 : 100	20 : 100	—
	b			33 : 100	4 : 3 : 3	7 : 1 : 1	6 : 0 : 0	—
					75 : 100	14 : 100	0 : –	—
97	a	6.08	304.0	15 : 12 : 10	7 : 5 : 5	8 : 6 : 5	8 : 8 : 7	—
					71 : 60	75 : 83	100 : 88	—
	b			80 : 83	6 : 3 : 2	8 : 3 : 3	6 : 2 : 1	6 : 0 : 0
					50 : 67	38 : 100	33 : 50	0 : –
Unweighted mean	a			60.4 : 87.6	58.8 : 82.0	43.8 : 92.6	48.8 : 77.6	—
	b				43.2 : 73.4	21.8 : 83.3	13.2 : 75.0	0 : –
Weighted mean	a			63.3 : 87.1	57.6 : 89.5	45.9 : 88.2	54.8 : 88.2	—
	b				40.6 : 84.6	22.2 : 75.0	13.8 : 66.7	0 : –
Mean number injected sperm (millions)	a			231.5	231.5	231.5	231.5	231.5
	b			231.5	57.9	14.5	3.6	0.9

fall at a fairly steady rate until complete infertility is reached at a dilution of 1/256 or a total of 900,000 injected sperm. It should be noted that fertility obtained with the pure sperm in this and the experiments listed in Table V was about 20% less than that obtained in the tests done earlier in the year. It may be, therefore, that repetition of the last experiment at a season when fertility is higher might raise the levels of fertility somewhat in all dilutions, although this probably would not change their relative positions. As long as the comparisons are made within experiments, valid conclusions can be drawn concerning the effects of number of injected sperm and dilution.

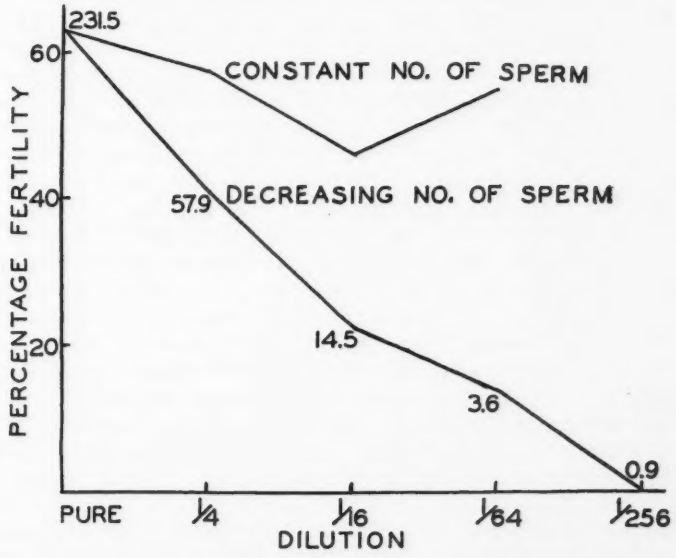

FIG. 2. *A comparison of the effect on fertility of injecting a constant or a decreasing number of sperm at various dilutions. Sperm serum as diluent. Number of injected sperm in millions is shown on the curves.*

In drawing general conclusions, therefore, one cannot be too dogmatic about the effects of sperm numbers on fertility. Quite possibly a small amount of fertility might be secured with as few as one million sperm in a more favorable season, and with larger numbers this might be correspondingly higher. However, it seems likely that any increase to be secured on repetition would manifest itself at the undiluted or slightly diluted levels. It is certain that enormous numbers of sperm, probably one hundred million or more, must be placed within the vagina of the hen before fertility will remain at a normally high level for a period of 10 days. When the number is reduced to fifty million, a noticeable drop in fertility occurs; when the number reaches one million, complete or nearly complete sterility ensues.

Effects on Hatchability

Thus far, fertility has been considered and although the percentage hatchability has been listed in each table, only the former has been discussed. Hatchability, as used in this study, refers to the percentage of fertilized eggs that hatch, and is independent of fertility in computation. The recorded evidence concerning the relation between fertility and hatchability is rather contradictory. Under certain circumstances there appears to be a correlation between them (Pearl and Surface (10)), and Knox (4) found that the simple correlation coefficient measuring the degree of association between the two was +0.59. In practical poultry work it is the general experience of the breeder that the number of dead embryos is inordinately high during periods of low fertility. Possibly, this might be due to a lack of physiological vigor whereby a high proportion of non-functional sperm resulted in fewer fertilized eggs, and whereby a lack of normal vitality of the functional sperm caused a higher death rate in the resultant embryos. Such an explanation presupposes the existence within the sperm of varying protoplasmic states that affect embryonic development. This is a somewhat novel concept, not necessary to explain a correlation between fertility and hatchability; the correlation can be explained more satisfactorily by assuming variations within the egg that influence impregnation as well as embryonic changes. The large amount of complex food matter in the hen's egg provides a wide range for variation, making it more probable that the egg controls the developmental processes. The first possibility has been offered, however, since the data are pertinent to it. For instance, since the synthetic diluent decreased the fertilizing power of the sperm, probably by rendering a certain proportion non-functional, it is reasonable to suppose that a sub-lethal effect has been produced on at least some of those remaining functional. This supposition receives considerable weight when it is remembered that the proportion of non-functional sperm increased with the degree of dilution (Table IV), which may mean that there are varying levels of susceptibility between individual cells in the same suspension.

One might expect, therefore, that in suspensions in which large numbers of sperm have become non-functional, the functional sperm might be so affected that many of the embryos that they conceived would die before hatching. Inspection of Tables III and IV, however, serves to dispel any such notion. Table III shows that one fertile egg of 87 set from the 1/128 dilution, and six of 97 set at the 1/32 dilution, hatched. Statistical analysis reveals no correlation between fertility and hatchability at the various levels. Therefore, it appears that sperm that survive unfavorable treatment *in vitro* do not manifest their experience by any vital effect on the embryos they subsequently conceive. While we cannot generalize on this statement and conclude that the physiological state of the male parent is not reflected in the hatchability of the egg, we can advance the tentative hypothesis that once fertility is accomplished, the history of the developing embryo is largely controlled by the chemical composition of the egg. Such a theory excludes the effect of heritable genes

that may influence the development of the embryo. It applies only to seasonal or environmental variations in hatchability and has broader implications bearing directly on the general problem of embryonic mortality. It follows, for instance, that when fertility is high and hatchability low, the cause lies in the egg and is independent of the sperm; when fertility is low and hatchability normal, the male is at fault because either non-functional or insufficient numbers of sperm are produced; and when both are low, the egg at least is at fault and the sperm may possibly be.

Discussion

One of the significant results of this study is the demonstration of the marked effect that the diluent has on the fertilizing capacities of the sperm. This suggests that similar effects must have influenced the results obtained by Walton (11) in the rabbit, and therefore, comparison of the data on the quantitative nature of fertility in the fowl (demonstrated with the sperm serum diluent) is not possible. However, a closer study of Walton's work shows that while the simple sodium chloride diluent that he used may have adversely affected the functional capacities of the rabbit sperm, the effect is far smaller than that of Milovanov's diluent on fowl sperm. The lowest dilution used by Walton was one part semen to ten parts diluent (*i.e.*, 1/10 in the terminology of this paper), and the series increased by multiples of 10 (*i.e.*, 1/10, 1/100, 1/1000, etc.). Although his results are tabulated in terms of sperm cells per unit volume, it appears that he secured fertility in some of the rather weak dilutions, at least as low as 1/10,000 and 1/100,000. At any rate, he secured no significant decrease in fertility until the total number of sperm injected was less than one million, and fertility ceased only when less than ten thousand were injected. When it is considered that these few sperm were suspended in as much as 3 cc. of fluid, there is little doubt that an enormously greater proportion of diluent was present in Walton's suspensions. As Milovanov's diluent is a more favorable fowl sperm diluent than normal saline, when judged by motility effects *in vitro*, it is clear that normal saline is either more satisfactory for rabbit sperm than for fowl sperm, or rabbit sperm is more highly resistant to unfavorable media of certain types.

Even admitting an adverse effect of the diluent in Walton's work—a possibility that he realized and referred to as "differential toxicity"—it is clear that the number required to ensure fertility in the rabbit is very much less than in the fowl. Optimum fertility in Walton's work was reached with about one million sperm, and in this work the indicated point is about one hundred million. Both minimum and maximum fertility in the rabbit seem to occur with only 1% of the numbers of sperm required to produce similar fertility levels in the fowl.

In this connection, however, it must be remembered that Walton injected a volume of 3 cc., whereas the present work is based on injections of 0.05 cc. While the volume may be relatively immaterial it seems possible that when the vagina of a rabbit is distended with a sperm suspension as in Walton's

work, some of the fluid might find a natural outlet by infiltration into the uterus and tubes. As the sperm would naturally be carried in their original concentration wherever the fluid penetrated in even the smallest amount, the transportation of the sperm would be aided. The exact mechanism of sperm transportation is still unknown; other factors in addition to the motility of the sperm are apparently involved (Hartmann (3), Parker (9)). It is generally agreed, however, that fewer sperm are encountered at successive levels of ascent within the Fallopian tubes of mammals, presumably because of the mechanism of transport. It can be seen that sperm introduced into the vagina or distal end of the uterus in concentrated form must find their way into the tubes by moving in the natural fluids. Thus they would be considerably handicapped compared with the same number suspended in a large bulk of fluid. Whether volume does assist fertilization depends on the penetration of the uterus and tubes by the injected sperm suspension, and therefore, on the level at which the natural mechanism begins to produce the usual thinning of sperm at successively higher levels. Presumably, volume might aid in the transportation of fowl sperm, as it appears to have done in the rabbit, but this has not been a factor in the present experiment, as the effect of sperm numbers on fertility (Table VI) was measured by injecting a volume of only 0.05 cc.

The fact that the quantitative basis of fertility in the fowl is on a much higher numerical level than in the rabbit is not surprising, in view of the comparative length, breadth and heavily ridged internal surface area of the oviduct of the fowl. Many more sperm would be required to seed the entire surface of this organ as thickly as the much smaller, although paired, mammalian analogue. This may explain the much higher density of sperm in fowl semen (about six million per cu. mm.) than in rabbit semen (one hundred thousand per cu. mm., Lloyd-Jones and Hays (5)).

Acknowledgment

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THE EGG-PRODUCING CAPACITY OF POPULATIONS OF *TRIBOLIUM CONFUSUM* DUV. AS AFFECTED BY INTENSIVE CANNIBALISTIC EGG-CONSUMPTION[1]

By John Stanley[2]

Abstract

Experiments are described in which adults of *Tribolium confusum* Duv. are maintained at 27° C., and 75% relative humidity in four different flour media: *(a)* ordinary whole wheat flour sifted through 76-mesh bolting cloth, *(b)* similar flour with from 30 to 135 *Tribolium* eggs per gm., *(c)* sifted whole wheat flour plus 3% of finely grourd wheat germ and *(d)* flour plus germ plus eggs.

It is shown that when large numbers of eggs are eaten, there is a serious decline in egg production unless wheat germ in excess is also present. This is believed to be due to a scarcity of certain accessory growth substances found in wheat germ but not to the same extent in eggs. When ground wheat germ is present, the beetles seem to do somewhat better in the presence of eggs, possibly because of a better water supply, obtained from the eggs.

Introduction

As a result of observations on some populations of *Tribolium confusum* in which the eggs were eaten as rapidly as laid, the writer suspected that the egg-laying rate of females in such populations was seriously reduced.

As the amount of flour consumed under these conditions of intensive egg eating is much less than normal, it seemed reasonable, in the light of the work of Sweetman and Palmer (2) on the accessory growth substances present in wheat germ and necessary to normal egg production, that this reduction in fecundity might be due to a lessened intake of these substances.

Accordingly, five experiments* to test this hypothesis are described below. The results support the above supposition.

Methods

All populations consisted of eight males and eight females, newly reared, and maintained in 32 gm. of medium at 27° C., and 75% relative humidity. Experiments Nos. 1, 2 and 3 were carried out in ordinary whole wheat flour which had been sifted four times through 76-mesh silk bolting cloth. In Expt. 1, the eggs were removed, counted and discarded every day, and the flour was changed every four days. The experiment was continued for approximately 33 days. The data are shown in Table I.

It is to be regretted that it was necessary to terminate Expt. 1 after 33 days, as this makes comparison with Expts. 2, 4 and 5, maintained for longer periods, somewhat less convincing. However, other data obtained in this

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Contribution from the Department of Biology, Queen's University, Kingston, Ontario, Canada, with financial assistance from the National Research Council of Canada.
[2] *Associate Professor of Biology, Queen's University.*

* *It has been suggested that duplicate experiments should have been done for each medium. It should be noted that 16 insects are used in each group, so that each experiment has a greater statistical significance than if only one insect were used. There is in a sense an internal replication, and provided that all experiments are subjected to substantially identical environmental conditions, the labor and expense of replicating experiments of this type with Tribolium is hardly justified.*

laboratory show that populations handled as in Expt. 1 do maintain their egg production relatively unimpaired for longer periods than 30 days. Several such tests provide the data of Table II.

In Expt. 2, an average of 30 eggs per gram was maintained in the flour for a period of approximately 35 days (Table III), by removing the flour and eggs every four days and replacing them by one-day-old eggs and fresh flour. Following this exposure to the eggs, the beetles were placed in egg-free flour and the egg production per 24 hr. was determined for approximately 16 days.

TABLE I

TOTAL NUMBERS OF EGGS PRODUCED IN EXPERIMENT NO. 1

Time, days	Eggs	Time, days	Eggs	Time, days	Eggs
0.00	0	10.77	560	21.77	1128
.87	42	12.10	618	22.92	1190
1.86	90	12.79	664	23.75	1238
3.10	164	13.96	716	24.75	1290
4.03	218	14.78	762	25.79	1336
5.03	260	15.90	810	26.89	1390
6.05	316	16.77	862	27.87	1434
7.01	370	17.76	914	28.76	1476
7.75	404	18.78	976	29.84	1534
8.91	462	19.85	1032	30.78	1586
9.78	512	20.97	1078	31.74	1638
				33.85	1688

TABLE II

EGG-PRODUCTION RATES OF BEETLES LIVING IN SUBSTANTIALLY EGG-FREE FLOUR

Test	Time, days	Egg production, eggs per 24 hr.
A	0	52.23
	23.00	48.16
	74.03	47.61
B	0	70.08
	15.74	69.35
	30.97	69.00
	45.74	65.37
	60.17	61.80
	75.50	55.11
C	0	64.34
	50.33	63.25
	101.75	56.70

In Expt. 3, after an initial study had determined an average rate of 80.81 eggs per 24 hr., the beetles were placed for approximately eight-day intervals in flour with 50 eggs per gram, maintained as above. After each eight-day interval the egg production was determined for 24 hr. in egg-free flour, and the procedure was repeated. The data are shown in Table IV.

For Expts. 4 and 5, 3% of finely ground wheat germ was added to the flour and mixed for 24 hr. in a ball mill with a reduced load of pebbles. It was found necessary to use only a few pebbles; otherwise the mass was ground rather than mixed, becoming sticky and difficult to sift.

In Expt. 4, the egg production was determined for each four-day interval (Table V), the flour being changed

TABLE III

DATA SHOWING COMPUTATION OF EGG-PRODUCTION RATES* FOR EXPERIMENT NO. 2

Time of test, days from start	Duration of test, days	Eggs produced	Rate, eggs per 24 hr.	Central time of test, days
34.96 to 38.94	3.98	68	17.08	36.95
38.94 to 43.07	4.13	91	22.03	41.00
43.07 to 47.75	4.68	82	17.52	45.41
47.75 to 50.83	3.08	66	21.43	49.29

* *Initial rate at T = 0, 68.65 per 24 hr.*

TABLE IV

DATA SHOWING COMPUTATION OF EGG-PRODUCTION RATES* FOR EXPERIMENT NO. 3

Time of test, days from start	Duration of test, days	Eggs produced	Rate, eggs per 24 hr.	Central time at test, days
9.98 to 10.97	.99	32	32.32	10.47
18.05 to 19.03	.98	29	29.59	18.54
25.92 to 27.92	2.00	63	31.50	26.92

**Initial rate at T = 0, 80·81 per 24 hr.*

TABLE V

TOTAL NUMBERS OF EGGS PRODUCED IN EXPERIMENT NO. 4

Time, days	Eggs	Time, days	Eggs	Time, days	Eggs
0	0	69.16	3603	132.15	5836
3.90	239	73.07	3781	136.28	5912
7.95	492	77.22	3950	140.16	5959
12.00	747	80.94	4117	144.25	6013
16.10	994	84.91	4288	148.16	6076
20.11	1242	88.95	4448	152.30	6131
24.12	1475	93.12	4600	156.49	6185
26.87	1628	96.93	4779	160.20	6208
32.99	1954	101.06	4958	164.07	6243
37.19	2186	105.02	5122	168.14	6264
41.06	2382	109.22	5297	172.31	6293
45.17	2588	113.08	5326	176.09	6322
49.11	2765	117.19	5495	181.28	6354
53.09	2938	120.13	5588	184.16	6362
57.11	3100	124.18	5677	188.23	6381
60.93	3261	128.09	5760		
65.13	3450	129.30	5783		

TABLE VI

DATA SHOWING COMPUTATION OF EGG-PRODUCTION RATES FOR EXPERIMENT NO. 5

Time of test, days from start	Duration of test, days	Eggs produced	Rate, eggs per 24 hr.	Central time of test, days
0 to 6.02	6.02	350	58.11	3.01
26.79 to 30.81	4.02	299	74.45	28.80
51.81 to 56.75	5.94	350	58.87	54.28
82.72 to 86.89	4.17	199	47.69	84.81
110.72 to 116.94	6.22	334	53.70	113.83
140.92 to 145.11	4.19	133	31.74	143.01
168.85 to 172.98	4.13	111	26.88	170.42
203.99 to 206.94	2.94	60	20.37	205.46

and the eggs discarded at each count. No extra eggs were added. The experiment was maintained for 188 days, when only six males and three females remained, and these were quite feeble. In Expt. 5 (Table VI), an average of 135 eggs per gram was maintained in the flour, except for a one-day test of egg production at approximately every 28 days. This concentration of eggs was obtained by adding all the eggs produced by a large stock culture of beetles. The number of eggs was estimated by weighing, and the mean computed at the end of the experiment. The number fluctuated from 80 to 190 per gram of flour. As eight males and eight females in 32 gm. of flour will eat eggs as rapidly as laid when the concentration is 30 eggs per gram, it is seen that the number used in this experiment would result in very intense egg eating. The writer is able to state, from other observations, that less than this number will result in the maximum possible egg consumption.

The resultant data were analyzed as follows: In Expts. 1 and 4, where repeated egg counts were available, cumulative egg curves showing the total production of eggs from the beginning of each experiment were plotted as in Figs. 1 and 3. Smooth curves were fitted to these by the Method of Least Squares, and the

resultant equations differentiated once to produce equations for the rate of egg production per 24 hr. From these equations the straight lines for Nos. 1 and 4, in Figs. 2, 4 and 5 were plotted.

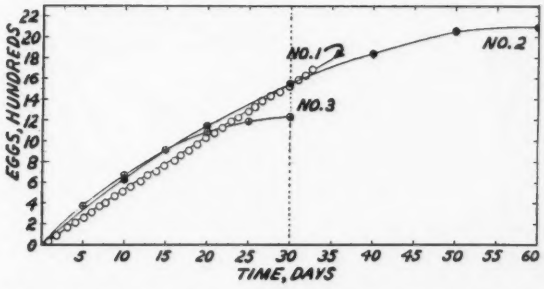

FIG. 1. *Curves showing the total number of eggs produced from the beginning of Experiments Nos. 1, 2 and 3. Nos. 2 and 3 are plotted from equations obtained by integrating the equations fitted by the Method of Least Squares to the data of Nos. 2 and 3 of Fig. 2. No. 1 is a Least Squares fit to the points shown.*

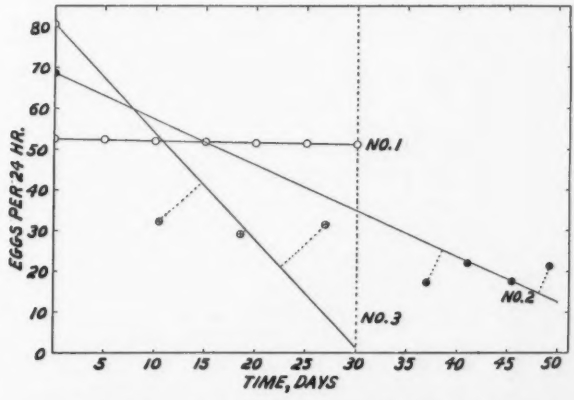

FIG. 2. *Straight lines showing rates of egg production per 24 hr. for Experiments Nos. 1, 2 and 3. No. 1 is plotted from the first differential of the equation fitted by the Method of Least Squares to the data of No. 1 in Fig. 1. Nos. 2 and 3 are Least Squares fits to the points shown, with a modification of the computations which forced them to go through the values 80.81 and 68.65 at $T = 0$ respectively, in order to show the decreases from these values.*

In Nos. 2, 3 and 5 the data were in the form of rates of egg production observed at rather wide intervals. In these, a rate equation was fitted by the Method of Least Squares, and from the equations the straight lines Nos. 2, 3 and 5 of Figs. 2, 4 and 5 were plotted. These equations were then integrated once to obtain the equations of the smoothed cumulative egg production, and from the latter equations the smoothed curves Nos. 2, 3 and 5 of Figs. 1 and 3 were plotted.

These smoothed curves are shown only for comparison with Curves Nos. 1 and 4 of Figs. 1 and 3, and are not highly accurate representations of the course of events in Experiments 2, 3 and 5. They are based on the straight lines Nos. 2, 3 and 5 of Figs. 2 and 4, which are straight only by virtue of the assumption that they may be so fitted.

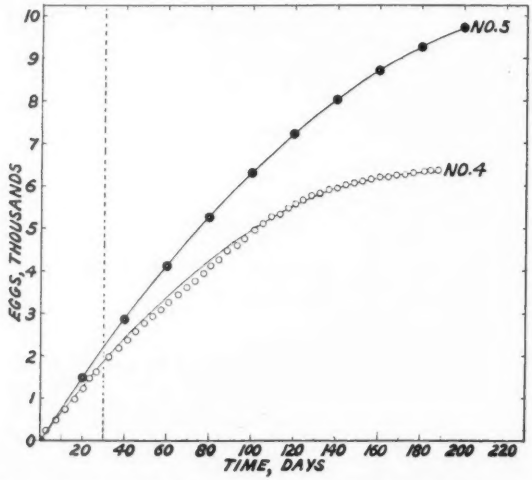

FIG. 3. *Curves showing the total number of eggs produced from the beginning of Experiments Nos. 4 and 5. No. 4 is a Least Squares fit to the points shown. No. 5 is plotted from an equation obtained by integrating the equation fitted by the Method of Least Squares to the data of No. 5 in Fig. 4.*

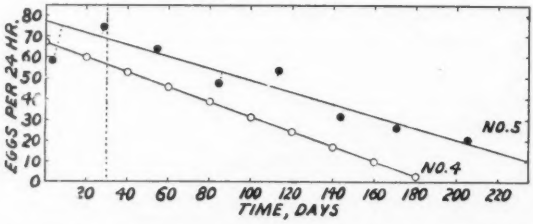

FIG. 4. *Straight lines showing the rate of egg production per 24 hr. for Experiments Nos. 4 and 5. No. 4 is plotted from the first differential of the equation fitted by the Method of Least Squares to the data of No. 4 of Fig. 3. No. 5 is a Least Squares fit to the points shown.*

It may be felt that the fit is not good in the straight lines Nos. 2, 3 and 5 of Figs. 2 and 4. In No. 5 of Fig. 4 this is due to experimental error. The experiments are not at all easy to carry out with accuracy, in spite of meticulous care with technique and excellent temperature control. In No. 2 it must be remembered that the effect of egg eating was no longer in operation when the egg production counts were made, so that production tended to remain substantially constant for the period studied. The discrepancies in

No. 3 may be due to the early death of the more susceptible females, leaving a hardy group whose egg-laying rate would probably have shown a steady downhill trend had the experiment been maintained for a longer period. In spite of this variation from a straight line, it was felt that, in comparison with Expts. 1 and 4, the same method of analysis should be applied throughout.

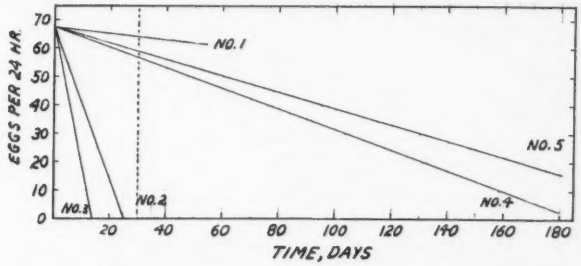

FIG. 5. *Summary of the lines showing egg-production rates per 24 hr. for all five experiments, with the lines plotted as if all experiments had started with an initial rate of 67.49, the same as No. 4.*

Discussion

It will be seen from Fig. 1 that the cumulative egg curve (No. 1) for Expt. 1 (ordinary flour without added eggs) rose steadily and smoothly with a very small diminution in egg production (see Fig. 2), the reduction being from 52.56 per day at T (time) = 0 to 51.44 at T = 30. It is clear that the beetles were doing well in this medium, especially as none died during the 30 days.

In Expt. 2 (ordinary flour plus 30 eggs per gram) the initial rate was higher (Figs. 1 and 2), as this lot of beetles was selected to have a somewhat higher than average rate. This resulted (Fig. 1) in the population producing a few more eggs than in Expt. 1 in spite of the fact that in Expt. 2 there was a marked decline in egg production, from 68.65 at T = 0 to 34.96 at T = 30 and to 12.50 at T = 50. (The latter is a value computed on the assumption that the effect of egg eating had continued.)

In Expt. 3 (Figs. 1 and 2) the results are similar, the reduced egg production being even more marked, consistent with the more intense egg eating.

That the females were adversely affected can be seen from the fact that all lived to more than 30 days in Expt. 1, only four were left at 30 days in Expt. 2, one of which was sterile, while only three were still alive at 30 days in Expt. 3. These surviving females were dissected; those from Expt. 1 were found to be normal, while those from Expts. 2 and 3 had suffered a remarkable degeneration of their reproductive tracts, amounting in two beetles to almost complete destruction of the ovaries and oviducts. Some experiments have been performed to see if such damaged females will recover, but all have died in a few days or weeks, even when transferred to a medium consisting of as much as 50% of wheat germ.

When *Tribolium* beetles are young they are of a clear reddish brown color with a faint superficial translucency, but as they age they become darker,

almost black in some cases. It was noticed that the beetles of Expts. 2 and 3 showed this change very early, and also seemed to become sluggish, with all the appearance of illness.

The results of Expts. 4 and 5, in which 3% of ground wheat germ was added to the flour, are entirely different. In Expt. 4 (Figs. 3 and 4) in which no eggs were added, the egg production falls smoothly and steadily, but not excessively, dropping from 67.49 at $T = 0$ to 56.74 at $T = 30$, and finally falling to 2.98 at $T = 180$, when only four males and one female were alive. These beetles were still quite active, but somewhat darkened. This is admittedly a more rapid decline in the early stages than occurred in Expt. 1, but the exceedingly small decline in egg production of the latter may have been due to the fact that as this was the first experiment, extraordinary care was used in removing the beetles from the flour. The flour was carefully turned over with a small brush, and the beetles dug out very gently. Time did not permit this method to be used in the other experiments, and the beetles were sifted out, although the writer had previously shown (1) that sifting was detrimental to egg production. However, a very gentle motion of the sieve was used, never violent enough to tear the beetles from their hold on the silk sifting-cloth.

In Expt. 5 in which a very large number of eggs was present, the decrease in egg production, so pronounced in Expts. 2 and 3, is almost absent (Figs. 3 and 4). The reduction was even less than in Expt. 4, in which no eggs were present. It is thought that this may have been due to a better supply of water, obtained from the eggs in Expt. 5. This supposition has no proof, but seems reasonable in view of the fact that *Tribolium* normally makes much of its water metabolically.

A summary of all five experiments is shown in Fig. 5, in which the straight lines for egg production per 24 hr. have been plotted with each experiment based on an initial egg-production rate of 67.49, the same as Expt. 4. This was chosen as the basic value because the insects of Expt. 4 were living in the standard medium now used for all experiments in the writer's laboratory. The drastic reduction in egg production caused by excessive egg consumption in the absence of a plentiful supply of wheat germ is clearly shown by the steep slopes of the lines for Nos. 2 and 3.

Acknowledgments

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